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14. ABSTRACT
The two goals of the grant are to 1) identify urine biomarkers of disease and therapeutic response in lupus nephritis (LN) patients, and 2) determine the pathophysiologic roles of glycosphingolipid (GSL) metabolism in renal cells. With respect to goal #1, we have identified, obtained, processed, and begun to measure N-glycosylated proteins and glycosphingolipids in 176 LN patients, 116 lupus patients without nephritis, and 114 healthy controls (HC). In addition, we measured GLSs and N-glycosylated proteins in urine and serum from a separate set of 20 LN patients and 20 healthy controls (HC) demonstrating extensive altered N-glycosylation and increased GSLs in the urine of LN patients and evaluated associations with biologic sex. With respect to goal #2, serum from these 40 individuals was used to stimulate human renal mesangial cells (hRMCs), demonstrating that the biologic sex of the cells had a significant impact on their proinflammatory response and corresponded with GSL levels. These results are included in a manuscript currently under review. We also collected extensive preliminary data over the past year in hRMCs and co-cultures of hRMCs and renal glomerular endothelial cells (hRGECs) analyzing cellular responses to external stimuli when reducing GSL levels using the drug eliglustat.

15. SUBJECT TERMS
Lupus nephritis, glycosphingolipid, N-glycosylation, N-glycan, Biomarkers, Mesangial cells

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INTRODUCTION

Glomerulonephritis affects more than half of patients with lupus. Despite use of mycophenolate and low dose cyclophosphamide, progression of the most aggressive forms of lupus nephritis (LN) to end stage renal failure remains high. The mediators of LN remain incompletely known and while many biomarkers have been identified to associate with renal disease in lupus patients, few have been shown to serve as markers of therapeutic response. Glycosphingolipid (GSL) levels in urine are significantly elevated in lupus patients with nephritis compared to healthy subjects or lupus patients without nephritis. Our lab supported by previous DoD funding, determined that levels of GSLs hexosylceramides (HexCers) and lactosylceramides (LacCers) were significantly higher in baseline (prior to treatment) urine samples from LN patients who failed to respond to standard of care mycophenolate mofetil treatment compared to patients that completely responded. *In fact, HexCers enhanced the prediction of complete response when included with clinical measures of eGFR and urine protein/creatinine ratio.* Additional preliminary data show significant differences between healthy subjects and LN patients in urine N-glycans and may be useful as biomarkers of therapeutic response. **We hypothesize that specific GLSs and N-glycans can be used to predict which lupus nephritis patients will respond to standard therapies, and that GSLs play key roles in the pathobiology of renal disease**. The objective of this proposal is to expand our previous studies of urine GSLs and identify urine N-glycans that together can be used clinically as a biomarker panel to predict treatment response. We also designed experiments to determine the role and mechanisms by which GSL metabolism modulates renal cell function and the pathogenesis of LN.

KEYWORDS

Lupus nephritis, glycosphingolipid, N-glycosylation, N-glycan, Biomarkers, Mesangial cells

ACCOMPLISHMENTS

- Major goals

Specific Aim 1, Major Task 1: Measure levels of HexCers and LacCers in urine samples from LN patients. Local IRB and HRPO approvals were obtained (subtask 1, completed). Individuals that met the inclusion criteria were identified and urine samples obtained from the MUSC biorepository (subtask 2, largely completed). The samples were aliquoted and most have been processed/prepared for analysis (subtask 3, underway). We measured GSLs in total urine in a subset of the LN samples and compared to the levels in their urine exosomes (subtask 4, underway). The analyses of the glycosphingolipids (subtask 4) will be performed once the N-glycans have been analyzed. Based on initial results, we will use total urine instead of urine exosomes (as done for our previous studies). Using total urine will be more straight-forward (requires less processing) and likely more clinically applicable. Additionally, for subtask 4 we plan to measure the lipids using MALDI-IMS first, which will allow us to measure levels of HexCers and LacCers as well as many other lipids simultaneously in the same sample. Differences in lipids observed by MALDI will then be confirmed by SFC/MS/MS at the MUSC Lipidomics core facility.

Specific Aim 1, Major Task 2: Measure levels of N-glycans in urine and serum samples from LN, lupus non-nephritis, & healthy subjects. Local IRB and HRPO approvals were obtained (subtask 1, completed). Samples from the same LN individuals as in Major Task 1 are being used in this Major Task and a separate aliquot was obtained (subtask 2, underway), which was provided to co-investigator Dr. Drake. Approximately half of those samples have been processed (subtask 3, underway), and N-glycans measured by MALDI-IMS (subtask 4, underway).

Specific Aim 2, Major Task 1: hRMC-hRGEC direct co-cultures. Experiments to determe optimal conditions for manipulating GSL levels in hRMCs and co-cultures for the studies proposed in subtasks 1 and 2 are underway. We identified three different lots of primary hRMCs that naturally have different levels of HexCers and LacCers and showed they exhibit significantly different responses to human sera. We also showed that treatment with the GSL synthesis inhibitor eliglustat reduces HexCers and LacCers in the hRMCs. Analyses of eliglustat-treated hRMCs and hRMC-hRGEC co-cultures are underway (subtask 2). Optimal conditions for treating hRMCs and hRGECs with lipids (subtask 1) has proved more challenging and are at a preliminary stage.

Specific Aim 2, Major Task 2: hRMC-hRGEC indirect (transwell) co-cultures. The subtask designed to test whether effects on hRMCs or hRGECs are indirect have not begun. Once optimal treatment conditions with inhibitors, lipids and stimulants are determine in Specific Aim 2, Major Task 2, we will begin experiments for this Task. We now have a process in place at our institution for obtaining glomeruli isolated from discarded human kidneys. Thus, as time permits, we will pursue obtaining intact human glomeruli and activate the cells as described for the co-cultures. These glomerular “3D” cultures could provide results that are more representative of the cell communication that occurs *in vivo*.

- Accomplished under the goals

The major activities completed in the first year of the grant includes: 1) IRB approvals were obtained, and most of the urine samples were identified (176 LN, 116 lupus without nephritis, 114 healthy controls), obtained, and aliquoted for the GSL and N-glycans analyses; 2) processing and N-glycan measures collected for most of the urine samples; and 3) determining conditions for reducing GSLs in hRMCs using the FDA-approved drug eliglustat. In addition, in the process of working out conditions for the N-glycan/GSL analyses of the urine and cell culturing conditions, we made some interesting observations that are the basis of a manuscript currently under review for publication and described below. Specific Aim 1, Major Tasks 1 and 2 are ahead or on schedule. Specific Aim 2 Major Tasks 1 and 2 started slowly as we were still determining the optimal experimental conditions, but these experiments are now on track and we also may have a more robust experimental approach to include 3D cultures (intact glomeruli isolated from human kidneys).

Specific Aim 1- Major Tasks 1 & 2: Our previously published results on GLS levels in urine extracellular vesicles (EVs) from human lupus patients suggested that GSLs can enhance the prediction of therapeutic response (Troyer et al., Metabolites, 2022). Because EV isolation is a lengthy process, we wanted to test if total urine was representative of what we observed in the urine EVs. If so, using urine would be more amenable to measure in the clinic. Therefore, we measured HexCers in the urine samples from a subset of the patients from whom we measured these GSLs in their urine EVs in our published study (Troyer et al., Metabolites, 2022). We also measured N-glycans in a subset of these patients to determine if N-glycan levels also correlate between their urine and urine EV samples. Indeed, we determined there is significant correlation of total HexCer levels (Fig. 1A) and of N-glycans (Fig. 1B) in urine compared to urine EVs.

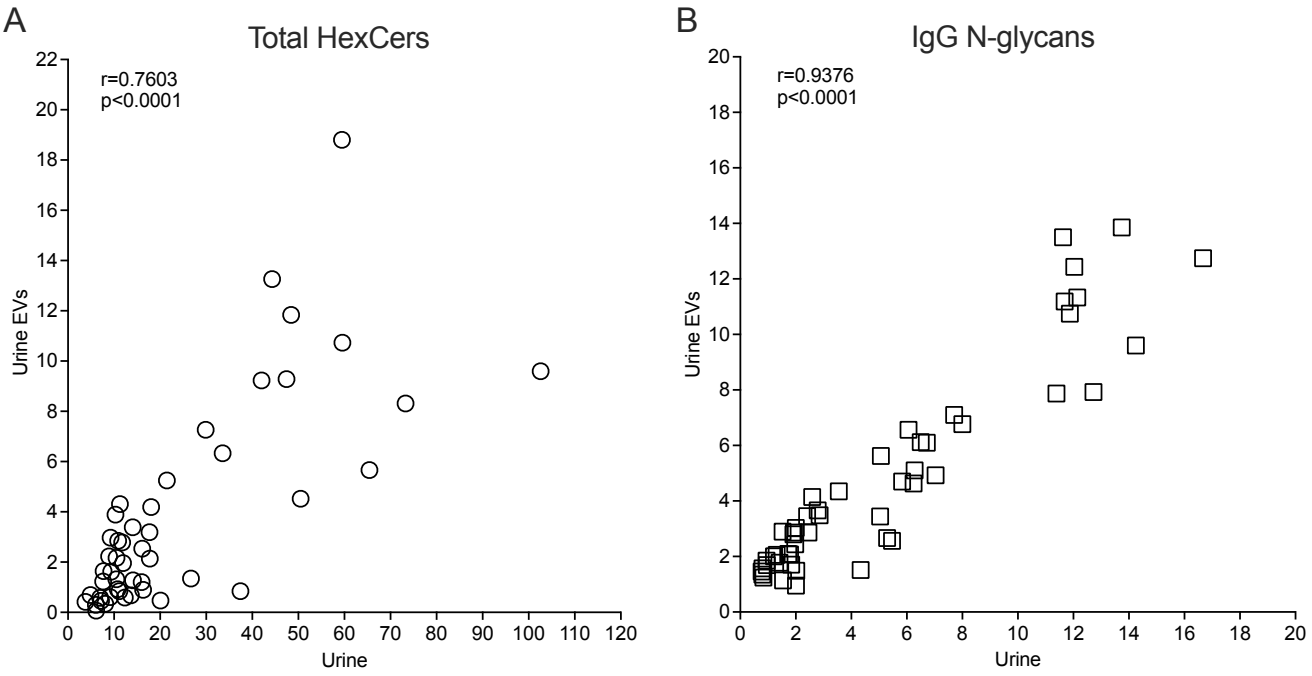


Fig. 1. GSLs and N-glycans levels in extracellular vesicles (EVs) or whole urine can be used to determine relative levels across groups. A) HexCers levels were measured in whole urine samples and EVs isolated from the same whole urine samples of 51 LN patients (23 complete responders, 28 non-responders). B) IgG (m/z 1485, 1663, 1809, 2122, 2435) N-glycan levels in whole urine samples and EVs isolated from the same whole urine samples of 10 LN patients (5 complete responders, 5 non-responders). Correlation analyses indicates significant correlation between levels of HexCers and levels of N-glycans in urine EVs and who urine.

As mentioned above, we have obtained 176 LN, 116 lupus without nephritis (SLE), 114 healthy controls (HC) urine samples. Most have been processed and about half of the samples have been analyzed for N-glycans. Thus far, analysis of 40 LN, 40 SLE, and 40 HC samples show clear separation of the LN group from the HC and SLE groups (Fig. 2). These analyses are ongoing and once data is collected from all samples, we will perform comparisons to include disease status, clinical measures, treatment response, etc. GSLs analyses will follow the N-glycan analyses.

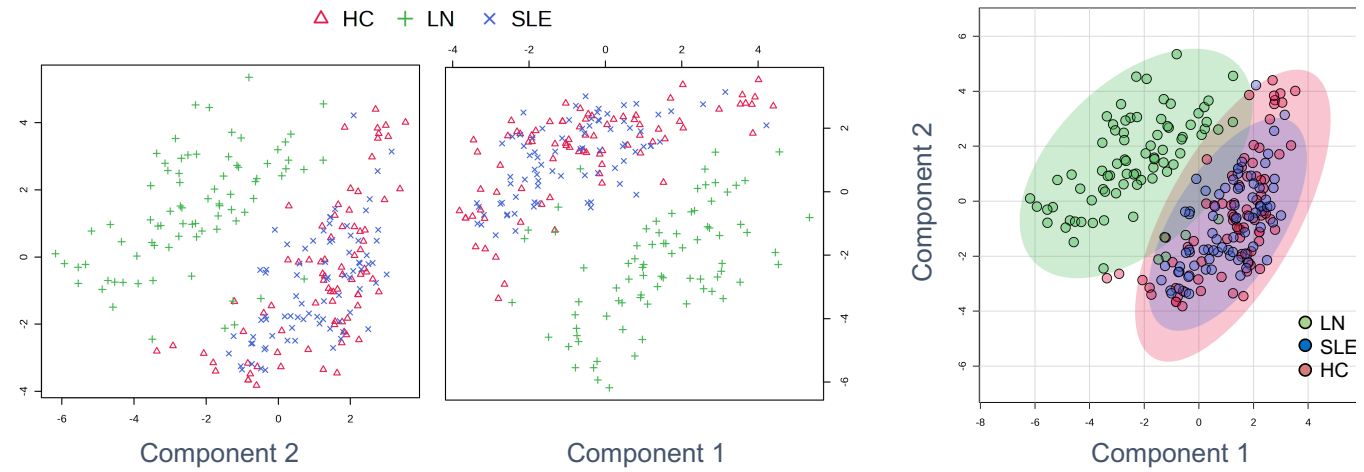


Fig. 2. Urine N-glycans can be used to identify LN patients from lupus patients without nephritis as well as HC. N-glycans were measured in urine from LN patients, lupus patients without nephritis (SLE) and HC. A sPLS-DA algorithm (left side) using the N-glycan peak signatures of the 3 groups was used to generate a 2D scores plot (right side), which shows clear separation of the LN patients from the SLE and HC individuals (40 individuals per group).

GSLs and N-glycans were measured in urine and serum samples from 20 LN patients (10 females, 10 males) and 20 HC (10 females, 10 males) to observe if GLSs or N-glycans in serum and urine from the same individuals are altered in a similar manner and if there are differences based on biologic sex. Indeed, urine GSL LacCers were elevated (Fig. 3A) in LN patients compared to HC. Although a significant difference across sexes was not observed (Fig. 3B), the ratio of LacCers in LN:HC for males was much higher than the ratios in females (Fig. 3C) suggesting a larger elevation occurs in males compared to females who develop LN. Of the 96 individual N-glycans detected in urine, 71 were significantly altered (Fig. 4A, top 10 differentially expressed N-glycans are shown), 3 were significantly different based on biologic sex (Fig. 4B), and the N-glycans associated with IgG were significantly skewed towards pro-inflammatory forms (Fig. 4C). Addition of any one of the significantly altered urine N-glycans to a model that included total urine LacCers and biologic sex improved discrimination between LN and HC, and inclusion of three of the N-glycans yielded perfect separation of LN and HC with an AUC of 1.0 (versus 0.870 for the model). In the sera, two of the major chain lengths of LacCers, but not the overall (total) levels of LacCers, were significantly increased (Fig. 5A) and the N-glycome was significantly altered (21 of 70 individual N-glycans detected) in LN compared to HC (Fig. 5B, top 10 differentially express N-glycans are shown). However, no differences were observed for the serum LacCers or any of the N-glycans between the sexes (data not shown). Only two of the IgG associated N-

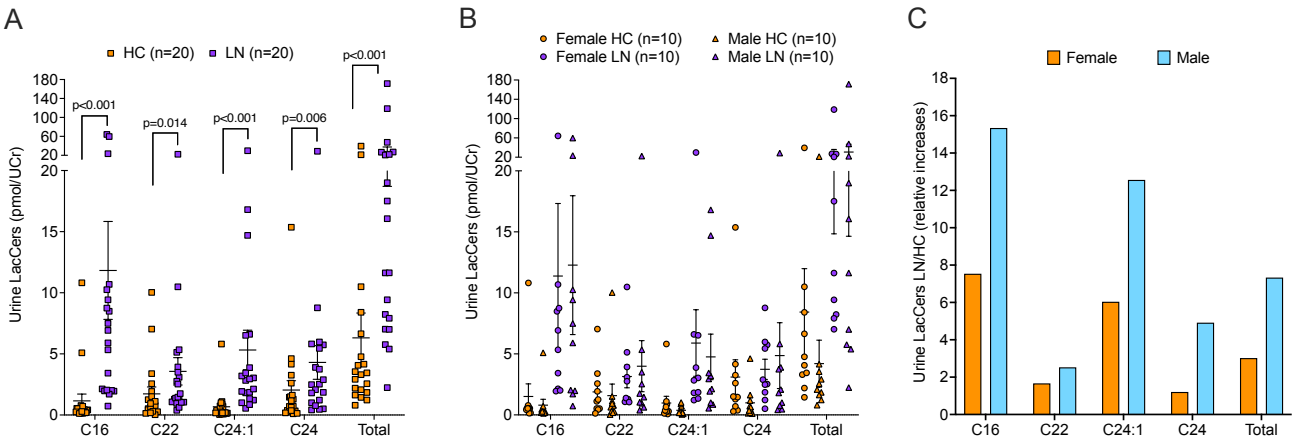


Fig. 3. Urine LacCers were significantly elevated in LN compared to HC; male LN patients exhibited a greater increase than female LN patients. LacCers were measured in urine of 20 LN patients and 20 HC; 10 females and 10 males in each group and normalized to urine creatinine, and LN was compared to HC (A) or female was compared to male across the LN and HC groups (B). While no significant differences were observed between males and females across the two groups, the ratio of HC to LN LacCers for females compared to males (C).

glycans were significantly different between LN and HC (Fig. 5C). Thus, more extensive significant changes are detected in the urine compared to the serum of LN patients when compared to HC.

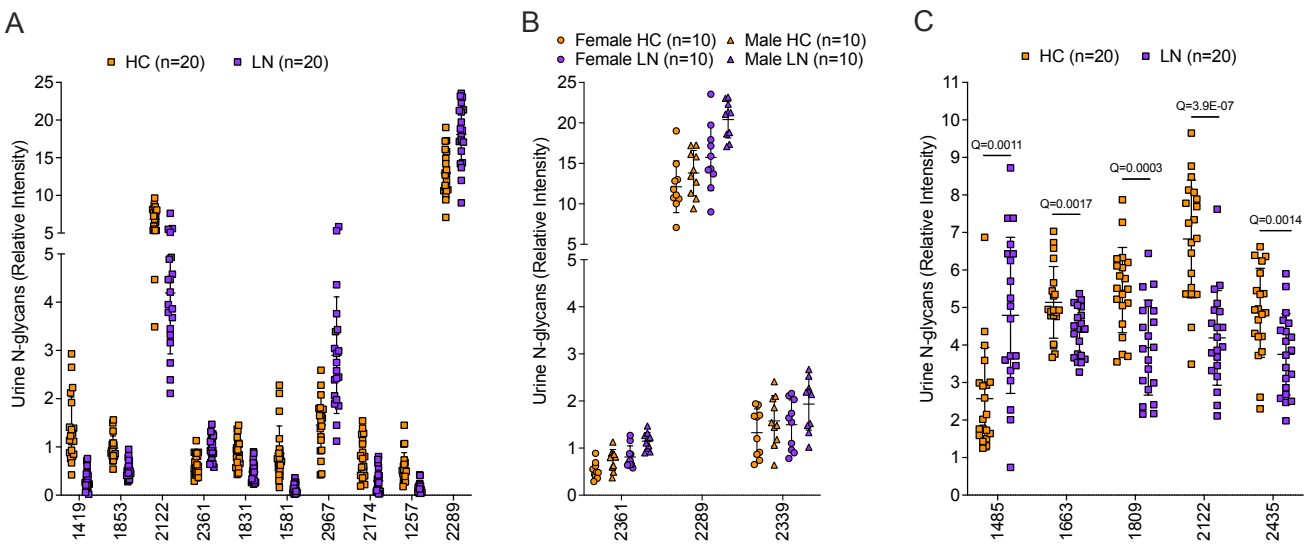


Fig. 4. Urine N-glycans were significantly altered in LN compared to HC with three significantly different by sex; IgG N-glycans were skewed with lower levels of the anti-inflammatory forms. N-glycans were measured in urine of the same individuals as in Fig. 2. A) 71 N-glycans were significantly different when comparing LN to HC (top 10 significantly different N-glycans are presented in the graph). B) 3 N-glycans were significantly different between females and males. C) IgG N-glycans were all significantly altered with higher levels of the more pro-inflammatory form (1485) and lower levels of the more anti-inflammatory forms (1663, 1809, 2122, 2435) in LN compared to HC.

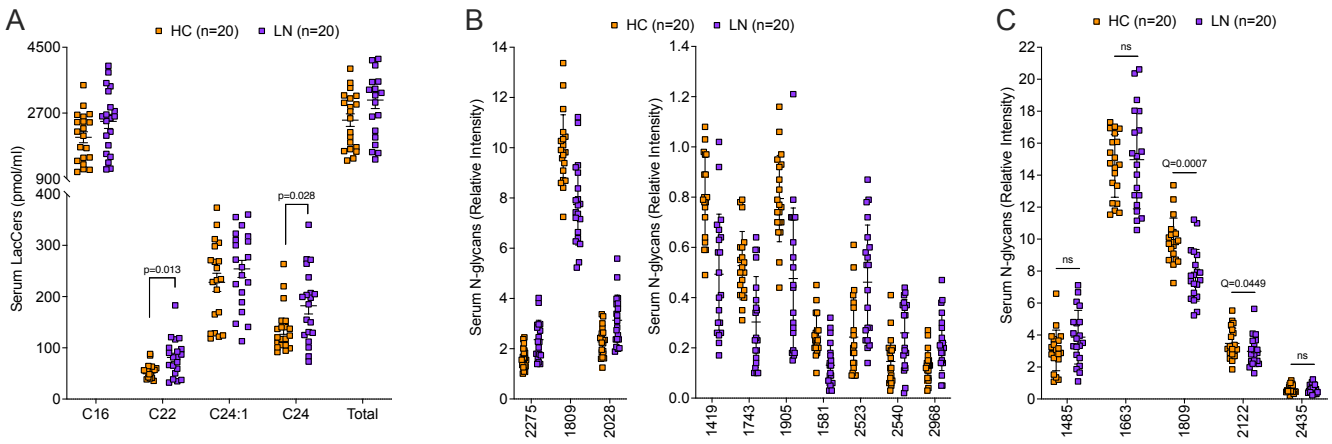


Fig. 5. Serum LacCers and N-glycans are not as different as those in urine in LN compared to HC. LacCers and N-glycans were measured in the serum of the same patients as in Figs. 1 and 2. A) 2 of the 4 major LacCer chain lengths were significantly elevated but overall LacCers levels were not different in LN compared to HC. B) 21 of the 70 N-glycans detected in the serum were significantly altered (top 10 significantly different N-glycans presented). C) 2 of the serum IgG N-glycans (compared to all the IgG N-glycans in urine) were significantly different between LN and HC; both were lower in LN and are the more anti-inflammatory forms.

Specific Aim 2- Major Tasks 1 & 2: Culture conditions are being optimized for co-culturing the hRMCs with the hRGECs with respect to external stimuli (LN serum, IL-1beta, and TNFα) using IL-6 as the initial downstream indicator of cell activation. In our preliminary data we observed a synergistic effect in the secretion of IL-6 in hRMCs-hRGECs co-cultures stimulated with TNFα, which was reduced when treated with eliglustat (Fig. 6). To determine the extent of GSL reduction by eliglustat we treated hRMCs with 20 or 200 nM eliglustat and observed that 20 nM provided ~5-fold reduction in HexCers (Fig. 7A) and LacCers (Fig. 7B). Additional conditions are being optimized (cell ratios, effects of serum starving, different external stimuli, and optimal eliglustat concentrations).

Experiments were also performed to identify additional downstream effects of activating hRMCs with various proinflammatory cytokines and LN vs HC serum by screening an array of 70 cytokines and chemokines. Results showed that CXCL5 and CCL5 were secreted at higher levels following stimulation with LN sera compared to HC sera (data not shown). To verify the array results, we used three lots of hRMCs from ScienCell Research Labs. Each lot was derived from a different individual. While all three hRMCs lots released significant amounts of CXCL5 and CCL5 in response to LN serum, no significant differences were observed between hRMCs stimulated with LN vs HC sera. LN and HC sera stimulated a ~2-5-fold higher release of CXCL5 and CCL5 from two of the lots (17544 and 20057) compared to the third lot (12445) (Fig. 8A-B and data not shown), which we determined had much lower levels of HexCers (Fig. 8C) and LacCers (Fig. 8D). The hRMCs with higher levels of GSLs also exhibited significantly higher intracellular Ca^{2+} flux (data not shown). Using 20 nM eliglustat in the highly-expressing GSL hRMCs (17544 & 20057) is anticipated to reduce their levels similar to those observed in the lower-expressing hRMCs (12445). We are currently analyzing the effects of eliglustat in the high-expressing GSL hRMC lot (17544) following various stimulants (LN serum and cytokines) to determine if reducing GSL

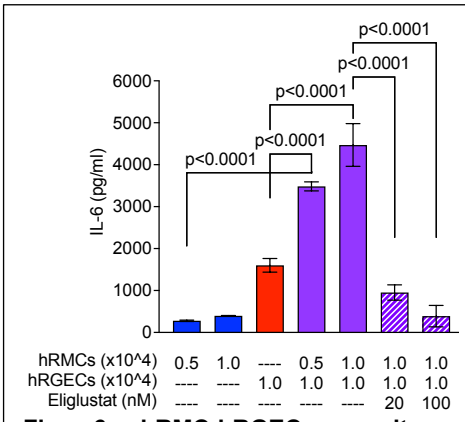


Fig. 6. hRMC-hRGEC co-cultures exhibit a synergistic response to TNFα that is blocked by eliglustat. hRMCs were seeded at the indicated number. Once the cells had plated down, hRGECs were seeded into the same wells. After the hRGECs plated down, the cells were pretreated with 20 or 200 nM eliglustat to reduce GSLs followed by stimulation with 100 ng/ml TNFα. Controls included hRMCs or hRGECs alone. Media was collected 24 hours after stimulation and IL-6 measured by ELISA.

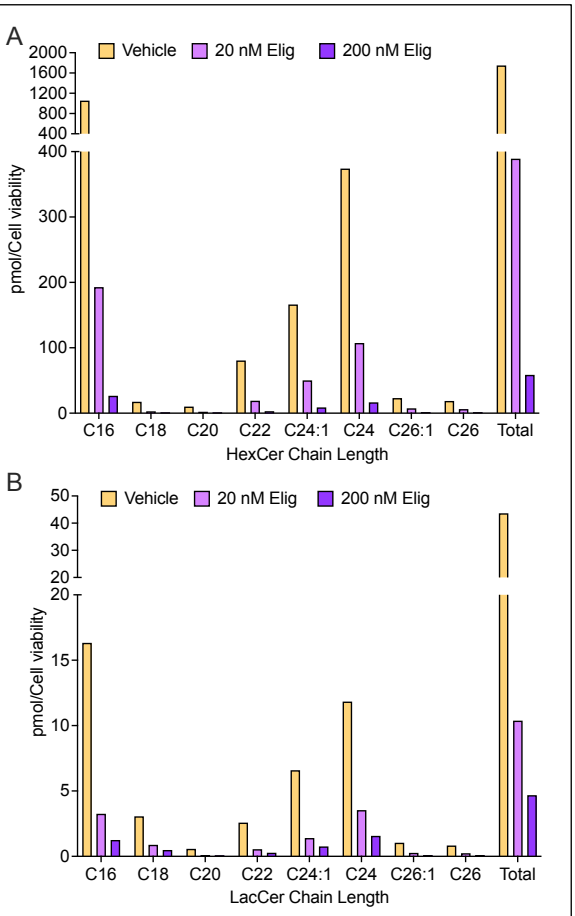


Fig. 7. Eliglustat effectively decreases GSLs in hRMCs. hRMCs were treated for 3 days with 20 nM or 200 nM eliglustat. Cells were collected and HexCers (A) and LacCers (B) were measured by the MUSC Lipidomics core facility and normalized to cell viability determined by alamar blue assay.

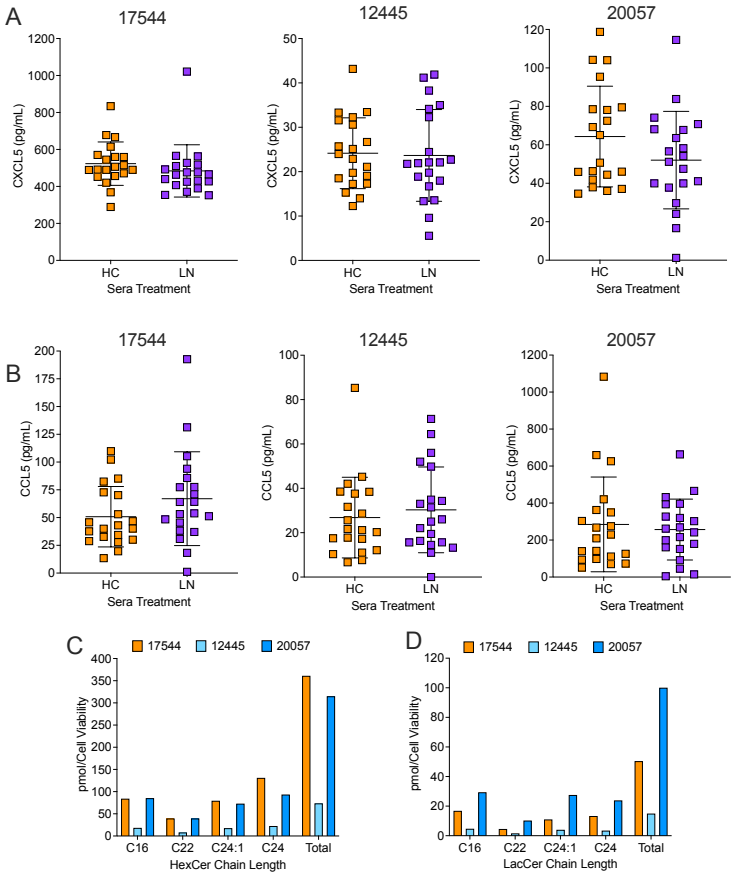


Fig. 8. hRMCs with high levels of GSLs, secrete significantly higher levels of CXCL5 and CCL5 than hRMCs with lower levels of GSLs. hRMCs were serum starved 3 hrs and stimulated with 5% LN or HC sera. CXCL5 (A) and CCL5 (B) were measured in the media and normalized to cell viability measured by alamar blue. HexCers (C) and LacCers (D) were measured in cells grown to ~90% confluency, and normalized to cell viability. hRMC lot #s 17544, 12445, and 20057 were each derived from one individual purchased from ScienCell Research Labs.

levels in this hRMC lot will decrease their proinflammatory response to multiple stimuli in monoculture and in co-culture with hGRECs.

- Opportunities for training and professional development

The PI has presented research findings from the funded studies at local programmatic seminar series and in her divisional research conference. The PI also had an abstract accepted for presentation at the upcoming American College of Rheumatology Convergence annual meeting November 10-15, 2023. The PI will meet with collaborators and other experts in the field to discuss approaches with plans to establish a collaboration with PIs who have repositories of urine from patient populations or clinical trials that have well-documented treatment response outcomes and clinic measures. We would like to obtain samples from another LN cohort to confirm results obtained with our current patient cohort. The PI and laboratory personnel also attend a weekly collaborative lupus meeting in which research or a journal article are presented alternating weeks. This provides an opportunity to discuss experimental results, pitfalls, alternative approaches, and next steps as well as stay current in the field.

- How were results disseminated to communities of interest

In addition to the presentations mentioned above, we have a manuscript under review (see appendix) that presents the results described for Figs. 3-5 and 8 in greater detail.

- Plans for the next reporting period to accomplish goals

Over the next year, we expect to complete the N-glycan analyses and most of the GSL analyses in Specific Aim 1 Major Tasks 1 and 2. Once all the raw data is collected, we will proceed with the statistical analyses to compare across the three groups, LN vs SLE vs HC, as well as within the LN group based on treatment response outcomes. Both N-glycans and GSLs will be considered in the statistical analyses to determine if a specific set of these molecules in combination with standard clinic measures will further enhance prediction of therapeutic response. We anticipate that the biostatistical analyses will be complete or nearly complete by the end of the next grant year. For Specific Aim 2 Major Task 1, we are focused on establishing optimal co-culture conditions with the various external stimuli, eliglustat, and mycophenolate (MPA). To provide a more focused approach to identifying mechanisms by which GSLs modulate renal cell function, we plan to measure gene expression changes via single-cell RNA-sequencing and changes in secreted protein levels using protein arrays. These approaches will identify signaling pathways of communication between hRMCs and hRGECs under conditions of high and low GSL expression in response to various inflammatory stimuli. We also plan to determine optimal conditions for siRNA knockdown of specific enzymes involved in GSL metabolism or other signaling pathways. For Specific Aim 2 Major Task 2, we will collect preliminary data from experiments with the indirect (transwell) co-cultures using the optimal conditions from the direct co-cultures. An added goal as time permits is to obtain glomeruli isolated from human kidneys (process for obtaining kidneys from discarded transplants is now in place by colleagues in the MUSC Nephrology division who will isolate and provide glomeruli). We will begin performing experiments with these “3D” cultures as they become available to optimize treatment conditions (based on those determined in our co-cultures) to compare with results from the “2D” co-cultures.

IMPACT

- Impact on the development of the principal disciplines of the project

We have not performed extensive statistical analyses of the N-glycan data, yet. However, based on our preliminary N-glycan data, we expect our results will provide important knowledge by identifying novel urine biomarkers that may predict which LN patients are unlikely to respond to standard therapies. This would be highly impactful to assist clinicians and patients in making informed decisions about therapeutic approaches resulting in better outcomes. Moreover, the IgG N-glycan analyses in urine (vs serum) could provide novel information about the pathophysiology of lupus and LN specifically.

- Impact on other disciplines

Nothing to report for this period

- Impact on technology transfer

Nothing to report

- Impact on society beyond science and technology

Nothing to Report

CHANGES/PROBLEMS

- Changes in approach and reasons for change

Nothing to report

- Actual or anticipated problems or delays and actions or plans to resolve them

The co-cultures proposed in Aim 2 have taken longer than anticipated to get optimized. Over the last month we have gained momentum on this aim and expect to be on track to achieve the originally planned subaims for the coming funding year.

- Changes that had a significant impact on expenditures

Nothing to report

- Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report

PRODUCTS

- Publications, conference papers, and presentations

One manuscript was peer-reviewed and is currently undergoing revisions (see appendix).

Bethany Wolf, Calvin R.K. Blaschke, Sandy Mungaray, Bryan T. Weselman, Mariia Stefanenko, Mykhailo Fedoriuk, Hongxia Bai, Jessalyn Rodgers, Oleg Palygin, Richard R. Drake, **Tamara K Nowling.** Metabolic markers and association of biological sex in Lupus Nephritis. International Journal of Molecular Sciences, *Under review*.

Many of our results to date will be presented at the upcoming American College of Rheumatology Convergence meeting in November 2023 and at the Society for Glycobiology annual meeting in November 2023 (see appendix for accepted abstracts).

Tamara Nowling, Bethany Wolf, Calvin Blaschke, Richard Drake, Sandra Mungaray, Mariia Stefanenko, Mykhailo Fedoriuk, Oleg Palygin, Hongxia Bai, Jessalyn Rodgers. Association of Biologic Sex, Glycosphingolipids, and the N-glycome in Lupus Nephritis and Renal Mesangial Cell Function. Nov. 2023. American College of Rheumatology Convergence Meeting, San Diego, CA.

Aaron O Angerstein, Sandra Mungaray, Danielle Beaufort, Tamara K Nowling, Richard R Drake. Identifying Urine N-glycans as Potential Biomarkers of Lupus Nephritis. Nov. 2023. Society for Glycobiology, Hawaii

- Website(s) or other Internet site(s)

Nothing to report

- Technologies or techniques

Nothing to report

- Inventions, patent applications, and/or licenses

Nothing to report

- Other products

Nothing to report

PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

- Individuals who worked on the project the past year

Name: Tamara Nowling, PhD
Project Role: PI
Research Identifier: ORCID ID: 0000-0002-1445-5415
Nearest person month worked: 2.4
Contribution to Project: Dr. Nowling directed planning of all experiments and analyses, met with and coordinated urine sample analyses with co-investigators, and assisted with and/or provided guidance with sample analyses. She also planned and directed personnel in performing cell culture experiments.

Name: Sandra Mungaray
Project Role: Research Specialist
Nearest person month worked: 6
Contribution to Project: Ms. Mungaray's main duties involved aliquoting urine samples for the N-glycan and lipid analyses, maintaining cell cultures, and performing cell culture experiments and downstream analyses (i.e. ELISAs). She has been assisting with establishing optimal conditions for the co-culture experiments in collaboration with co-investigator Dr. Jim Oates' lab. Ms. Mungaray was also instrumental in the extension of the proposed studies investigating the association of biologic sex in GSLs and N-glycans with respect to biomarker identification or role in hRMC activation.

Name: Danielle Beaufort, MS
Project Role: Research Specialist
Nearest person month worked: 4
Contribution to Project: Ms. Beaufort was hired 5 months ago and has assisted with aliquoting/processing urine samples for the N-glycan and lipid analyses and with the cell culture experiments. Following additional training, she is now assisting with performing the co-cultures experiments and downstream analyses.

Name: Richard Drake, PhD
Project Role: co-I
Nearest person month worked: 0.48
Contribution to Project: Dr. Drake performed the MALDI-IMS analyses to measure N-glycans on urine and serum samples.

Name: Jim Oates, MD
Project Role: co-I
Nearest person month worked: 0.21
Contribution to Project: Dr. Oates identified and obtained the urine and serum samples from the biorepository for the studies.

- Change in the active or other support of the PD/PI or senior/key personnel since last reporting period

Tamara Nowling: Since the awarding of this grant, Dr. Nowling began effort on three new studies:

1) R41DK135167 NIH Nowling (subaward PI) 9/15/23-9/14/24
Glycan biomarker panels in liquid biopsies for predicting treatment response in lupus nephritis
Goal(s): establish the innovative GlycoTyper platform as readily adaptable in the clinic for monitoring treatment response by analyzing the glycome in biofluids from lupus patients treated with standard-of-care therapies. The GlycoTyper platform will be used to validate N-glycan profiles (previously identified by established imaging methods, i.e. MALDI-IMS) in urine samples from lupus subjects with respect to therapeutic response longitudinally. Aim 2 will identify IgG specific N-glycan profiles in patient matched serum and urine samples with respect to therapeutic response. Together these aims will develop a clinically viable assay specifically for monitoring treatment response in lupus nephritis patients (to be called GlycoTyper-LNTx) that is readily adaptable to the clinic.
Role: PI (3.0 CM)
Overlap statement: There is no budgetary overlap with the DoD grant.

2) South Carolina Clinical & Translational Research Institute, MUSC Zhang (PI) 4/1/23-3/31/24
Targeting the transcription factor FLI-1 to treat neuropsychiatric lupus

Goal(s): demonstrate that the transcription factor FLI-1 modulates brain inflammation by regulating the expression of cytokine genes in microglial cells, and determine if inhibiting FLI-1 pharmacologically will improve neuropsychiatric manifestations of disease in a lupus prone mouse strain.
Specific Aims: Aim 1) Examine the role of FLI-1 in regulating inflammatory cytokines and NEU1 in microglial cells. Aim 2) To test the hypothesis that suppressing FLI-1 can ameliorate neuropsychiatric manifestations in lupus prone mice.
Role: co-I (no salary support)
Overlap statement: There is no budgetary overlap with the DoD grant.

3) I01CX001248 US Dept of Veterans Affairs Oates (PI) 6/1/22-5/31/26
Targeting Pathogenic Endothelial Dysfunction in Systemic Lupus Erythematosus
Goal(s): to demonstrate the most effective treatment indications, pharmacodynamic markers of response, and alternate or adjunctive targets to reduce inflammatory ECD in LN and other diseases of endothelial dysfunction in Veterans.
Role: co-I (1.2 CM)
Overlap statement: There is no budgetary overlap with the DoD grant.

Richard Drake: Since the awarding of this grant, Dr. Drake began effort on 3 new studies (#1-3) and effort on 3 studies ended (#4-6):

1) R41DK135167 NIH Nowling (subaward PI) 9/15/23-9/14/24
Glycan biomarker panels in liquid biopsies for predicting treatment response in lupus nephritis
Goal(s): establish the innovative GlycoTyper platform as readily adaptable in the clinic for monitoring treatment response by analyzing the glycome in biofluids from lupus patients treated with standard-of-care therapies. The GlycoTyper platform will be used to validate N-glycan profiles (previously identified by established imaging methods, i.e. MALDI-IMS) in urine samples from lupus subjects with respect to therapeutic response longitudinally. Aim 2 will identify IgG specific N-glycan profiles in patient matched serum and urine samples with respect to therapeutic response. Together these aims will develop a clinically viable assay specifically for monitoring treatment response in lupus nephritis patients (to be called GlycoTyper-LNTx) that is readily adaptable to the clinic.
Role: co-I (0.24 CM)
Overlap statement: There is no budgetary overlap with the DoD grant.

2) R01AG078702 Sun, Bendall, Angel, Drake (Multi-PI) 9/15/2022 – 8/31/2027
Deciphering the Glycan Code in Human Alzheimer's Disease Brain
Goals: The major objective of this study is to systematically resolve cellular and extracellular origins of perturbed complex carbohydrate metabolism using state-of-the-art single cell technologies. We will achieve this through synergistic integration of multi-parameter single cell mass spectrometry imaging methodologies. Aim 1. Define complex carbohydrates with clinical course and disease progression in patient samples. Aim 2. Interrogate cellular and extra-cellular architecture in normal and AD patient samples. Aim 3. Multimodal integration to track cellular and extracellular origins of complex carbohydrate perturbation in AD. This study will provide critical new information regarding ideal cell-, region- and temporally-specific opportunities for therapeutic modulation of AD.
Role: co-PI (0.12 CM)
Overlap statement: There is no budgetary overlap with the DoD grant.

3) R01 CA282022 Ippolito and Kim (PI) 7/15/23-6/30/28
Identifying lethal prostate cancer at diagnosis with advanced proteoglycomic, radiomic, and genomic approaches
Goals: This proposal advances the use of a clinical magnetic resonance imaging (MRI) sequence, diffusion basis spectral imaging (DBSI), that can detect structural and cellular changes in the PCa microenvironment (e.g., stroma, inflammation, tumor), that cannot otherwise be determined with conventional mpMRI. In parallel, our team has discovered a panel of extracellular proteoglycomic biomarkers in lethal forms of PCa (i.e., fucosylated glycans and modified collagens—"FuCol" biomarkers) with MALDI mass spectrometry imaging of histologic specimens. We hypothesize that MALDI-detected proteoglycomic markers, expressed as the FuCol score, are associated with structural and metabolic changes in lethal PCa that can be visualized with DBSI to better identify aggressive, potentially lethal PCa at the time of diagnosis. Aim 1:To validate our FuCol score as a predictor of disease recurrence and metastasis in a large institutional biorepository. Aim 2: To enroll a prospective cohort of prostatectomy patients to develop "Diffusion Molecular Imaging (DMI)"; an AI-driven tool that generates in vivo FuCol scores using in vivo DBSI as its input prior to prostatectomy. Aim 3: To develop an augmented risk

prediction model that incorporates novel DBSI imaging, the clinical Decipher genomics platform, and conventional clinical metrics to better predict lethal disease at prostatectomy.
Role: Co-I (0.18 CM)

4) W81XWH-17-1-0643 Wu and Drake (PIs) 09/30/2017 – 09/30/2022
A Novel Serum and Tissue Immunoglycomic Biomarker Panel to Distinguish Progressive PCa
Goals: The goals are to combine immune and glycan molecular markers from prostate tissues and serum to improve prediction of biochemical recurrence and/or variant neuroendocrine-like progressive prostate cancers. Aim1. Determine the sensitivity and specificity of tissue MIC and serum sMIC in predicting BCR and/or variant (NE-like) progressive PCa; Aim 2. Identify and evaluate clinically and biologically significant N-glycan panels in immune cell infiltrate in tissue and serum associated with BCR and/or NE-like PCa; Aim 3. Validate prognostic capacity of the identified panel of serum iomarkers.
Role: PI (0.6 CM)

5) R01 CA212409 Wu, Abduhar, Drake (Multi-PIs) 12/04/2017 – 11/30/2022
Tumor immune and glycan biomarkers for progressive prostate cancer
Goals: The goals of this multi-investigator study are to determine the sensitivity and specificity of tissue MIC and serum sMIC and tissue/serum glycan panels in predicting biochemical recurrence in a large cohort of Gleason 6 and 7 prostate cancer samples.
Aim 1. Determine the sensitivity and specificity of tissue MIC and serum sMIC in predicting BCR.Aim 2. Identify and evaluate clinically and biologically significant N-glycan panels in tissue and serum associated with BCR. Aim 3. Determine the prognostic capacity of serum and tissue biomarker panel. Aim 4. Validate prognostic capacity of the identified panel of serum biomarkers.
Role: PI (1.8 CM)

6) U01 CA242096 Drake, Angel, Mehta (Multi-PIs) 08/01/2019 – 07/31/2022
Simplified Glycan Profiling Workflows of Captured Immune Glycoproteins and Cells
Goals: The goals of the grant are to develop rapid glycan analysis workflows using basic MALDI-TOF MS instrumentation common to most institutions. Slide-based analysis of N-glycans from immunoglobulin classes, immune cells and cells grown in culture on slides is emphasized. SA1. Development of an on slide method for glycan analysis of immunoglobulin subtypes: SA 2. Development of Glyco-Cell Typer as applied to immune cell sub-types. SA 3. Analysis of cultured cells on slides for direct glycan measurements.
Role: PI (1.8 CM)

Jim Oates: Since the awarding of this grant, Dr. Oates began effort on two new studies:

1) R41DK135167 NIH Nowling (subaward PI) 9/15/23-9/14/24
Glycan biomarker panels in liquid biopsies for predicting treatment response in lupus nephritis
Goal(s): establish the innovative GlycoTyper platform as readily adaptable in the clinic for monitoring treatment response by analyzing the glycome in biofluids from lupus patients treated with standard-of-care therapies. The GlycoTyper platform will be used to validate N-glycan profiles (previously identified by established imaging methods, i.e. MALDI-IMS) in urine samples from lupus subjects with respect to therapeutic response longitudinally. Aim 2 will identify IgG specific N-glycan profiles in patient matched serum and urine samples with respect to therapeutic response. Together these aims will develop a clinically viable assay specifically for monitoring treatment response in lupus nephritis patients (to be called GlycoTyper-LNTx) that is readily adaptable to the clinic.
Role: co-I (0.24 CM)
Overlap statement: There is no budgetary overlap with the DoD grant.

2) COMETS-PPG MUSC Oates/Tsao/Palygin (multi-PI) 3/1/23-2/28/25
Oxidative and nitrosative stress in kidney damage
Goal(s): to search for new targets beyond the immune system to prevent and treat the severity of tubulointerstitial lesions in lupus nephritis, and gain knowledge on mechanisms of kidney damage mediated by the NCF1-H90 variant.
Role: Co-PI (0.21 CM)
Overlap statement: There is no budgetary overlap with the DoD grant

- Other organizations involved as partners

Nothing to report

SPECIAL REPORTING REQUIREMENTS

N-Glycans and Glycosphingolipids as Biomarkers and Modulators of Lupus Nephritis
LR210063, Annual Technical Report
W81XWH-22-1-0330

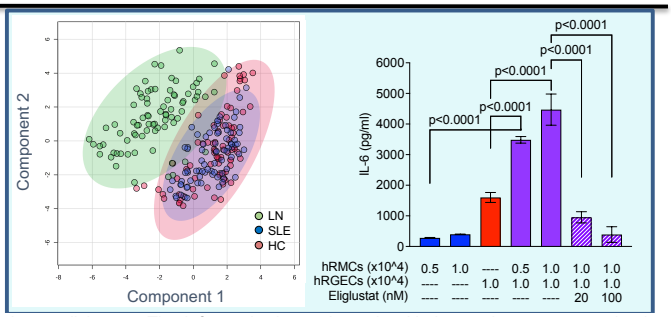
PI: Tamara Nowling **Org:** Medical University of South Carolina **Award Amount:** $750,000

Study/Product Aims

• Aim 1: Identify urine biomarkers of disease and therapeutic response in lupus nephritis (LN) patients
• Aim 2: Determine the pathophysiologic roles of glycosphingolipid (GSL) metabolism in renal cells
The goal of Aim 1 is to demonstrate that changes in the urine glycome profiles are more informative biomarkers of therapeutic reponse than current clinical measures. The goal of Aim 2 is to identify the roles of GSLs in renal cells in their response to inflammatory stimuli or their contributions to inflammation in the kidney.

Approach

Matrix assisted laser desorption/ionization imaging mass spectrometry (MALDI-IMS) used to measure changes in glycoproteins and glycolipids (glycome) of stored urine samples from lupus patients, and compared to clinical measures of disease to determine if the urine glycome can predict therapeutic response. Primary human renal cells manipulated to express varied levels of GSLs are exposed to inflammatory stimuli and changes in cell function and gene expression assessed to identify how GLSs impact cell function and contribute to disease.



Accomplishment: The left graph shows that urine N-glycan signatures can clearly distinguish lupus patients with nephritis (LN) patients from those without nephritis (SLE) and from healthy controls (HC) (Aim 1). The right graph shows that co-culturing primary human mesangial cells (hRMCs) and glomerular endothelial cells (hRGECs) results in a synergistic response to TNFalpha which was inhibited by the drug eliglustat, which reduces GSLs (Aim 2).

Timeline and Cost

Activities	Y1	Y2	Y3	
Measure GSLs in urine samples				
Measure N-glycans in urine samples				
hRMC-hRGEC co-culture analyses				
hRMC-hRGEC transwell culture analyses				
Estimated Budget ($K)	**$236,914**	**$248,553**	**$264,532**	

Updated: (02-22-2024)

Goals/Milestones

CY23 Goals – Measure GSLs and N-glycans in patient urine samples; Examine pathophysiologic roles of GSLs in renal cells
☑ Obtain IRB approval; obtain stored/archived urine samples and process for analyses; begin MALDI-IMS sample analyses
☑ Establish renal cell co-cultures; determine optimal conditions for reducing GSLs with the drug eliglustat
CY24 Goals – Measure GSLs and N-glycans in patient urine samples; Examine pathophysiologic roles of GSLs in renal cells
☐ Complete MALDI-IMS analyses of urine glycome comparing LN to SLE & HC; perform urine glycome analyses in treatment response patient samples
☐ Continue analyses of renal co-cultures with respect to changes in the cellular glycome; identify pathways to further pursue
CY25 Goals – Measure GSLs and N-glycans in patient urine samples; Examine pathophysiologic roles of GSLs in renal cells
☐ Complete all MALD-IMS analyses and perform statistical analyses
☐ Complete renal cell co-culture assays and analyses
Comments/Challenges/Issues/Concerns N/A
Budget Expenditure to Date
Projected Expenditures for Y1: $236,914
Actual Expenditures for Y1: $163,031

APPENDICES

- Bethany Wolf, Calvin R.K. Blaschke, Sandy Mungaray, Bryan T. Weselman, Mariia Stefanenko, Mykhailo Fedoriuk, Hongxia Bai, Jessalyn Rodgers, Oleg Palygin, Richard R. Drake, **Tamara K Nowling.** Metabolic markers and association of biological sex in Lupus Nephritis. International Journal of Molecular Sciences, *Under review*.
- Tamara Nowling, Bethany Wolf, Calvin Blaschke, Richard Drake, Sandy Mungaray, Mariia Stefanenko, Mykhailo Fedoriuk, Oleg Palygin, Hongxia Bai, Jessalyn Rodgers. Association of Biologic Sex, Glycosphingolipids, and the N-glycome in Lupus Nephritis and Renal Mesangial Cell Function. Nov. 2023. American College of Rheumatology Convergence Meeting, San Diego, CA. (Abstract, presentation)
- Aaron O Angerstein, Sandra Mungaray, Danielle Beaufort, Tamara K Nowling, Richard R Drake. Identifying Urine N-glycans as Potential Biomarkers of Lupus Nephritis. Nov. 2023. Society for Glycobiology, Hawaii (Abstract, presentation)

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Metabolic markers and association of biological sex in Lupus Nephritis

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Abstract: Lupus nephritis (LN) is a serious complication for many patients who develop systemic lupus erythematosus, which primarily afflicts women. Our studies to identify biomarkers and the pathogenic mechanisms underlying LN will provide a better understanding of disease progression and sex bias, and lead to identification of additional potential therapeutic targets. The glycosphingolipid lactosylceramide (LacCer) and N-linked glycosylated proteins (N-glycans) were measured in urine and serum collected from LN and healthy control (HC) subjects (10 females and 10 males in each group). The sera from the LN and HC subjects were used to stimulate cytokine secretion and intracellular Ca^{2+} flux in female- and male-derived primary human renal mesangial cells (hRMCs). Significant differences were observed in the urine of LN patients compared to HCs. All major LacCers species were significantly elevated and differences between LN and HC were more pronounced in males. 72 individual N-glycans were altered in LN compared to HC; three N-glycans were significantly different between the sexes. In hRMCs, Ca^{2+} flux, but not cytokine secretion, was higher in response to LN sera compared to HC sera. Ca^{2+} flux, cytokine secretion, and glycosphingolipid levels were significantly higher in female- compared to male-derived hRMCs. Relative abundance of some LacCers and hexosylceramides were higher in female- compared to male-derived hRMCs. Urine LacCers and N-glycome could serve as definitive LN biomarkers and likely reflect renal disease activity. Despite higher sensitivity of female hRMCs, males may experience greater increases in LacCers, which may underscore worse disease in males. Elevated glycosphingolipid metabolism may poise renal cells to be more sensitive to external stimuli.

Keywords: glycosylation, N-glycan, glycosphingolipid, lupus nephritis, mesangial cell, sex bias, biomarker.

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1. Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease in which the immune system can attack a variety of organs. Nephritis is a major complication of lupus

that occurs in greater than 50% of SLE patients. SLE also exhibits a strong sex bias occurring 9-10 times more frequently in females than males [1]. The underlying mechanisms involved in the development of nephritis in SLE patients is not completely known, nor is the sex bias in disease understood. While many studies have focused on understanding changes in the levels of genes or proteins, few have investigated the changes in lipids or glycosylation with respect to disease or sex bias.

Glycosphingolipids (GSLs) are neutral lipids synthesized from ceramide by addition of galactose or glucose to generate galactosylceramides (GalCers) or glucosylceramides (GlcCers), which together make up hexosylceramides (HexCers). Lactosylceramides (LacCers) are generated from GlcCers. These GSLs are involved in a wide array of functions in most cell types, including proliferation, apoptosis, and signal transduction [2-6]. We previously demonstrated in a small cohort of subjects that LacCers were significantly elevated in the urine of lupus patients with nephritis compared to lupus patients without nephritis or healthy subjects [7]. Differences in these GSLs were not observed in the serum. While sex differences of the circulating lipidome were recently reported, GSLs (GlcCers and LacCers) were not included [8]. To our knowledge, quantification of circulating or urine GSLs with respect to sex in healthy subjects, or to sex and disease in SLE patients, has not been reported.

Similar to GSLs, N-linked glycosylation of lipids and proteins plays an important role in mediating many different cellular functions including cell interactions and signal transduction. N-linked glycosylation can modulate the activity of proteins including IgG effector function [9]. While changes in the N-glycome are observed in many inflammatory or autoimmune diseases [10,11] including lupus [12-17], global changes in the N-glycome associated with disease or with biologic sex in lupus nephritis are unknown.

In this study, we analyzed differences in the levels of LacCers and N-glycosylated proteins (N-glycans) in urine and serum with respect to disease status and biologic sex. We show that all major LacCers in the urine, but only two major LacCers in the serum, were significantly elevated in LN patients compared to HC subjects in this study cohort. Although no differences were observed in overall levels of urine or serum LacCers with respect to biologic sex, a greater increase in urine LacCers was observed in males when comparing LN to HC. We observed that 75% of the urine N-glycans and 30% of the serum N-glycans were associated with disease status. Three of the urine N-glycans were associated with biologic sex. Activation of primary human renal mesangial cells (hRMCs) to the human sera was observed by measuring intracellular Ca^{2+} flux and cytokine release. Ca^{2+} flux was significantly higher in response to serum from LN patients compared to HC subjects. Intracellular Ca^{2+} flux in hRMCs derived from a female donor was more sensitive and the cells released 2-10-fold higher levels of cytokines in response to sera compared to hRMCs derived from a male donor. Interestingly, the GSLs levels were higher in the female-derived hRMCs compared to the male-derived hRMCs, and may contribute in part to the hyper-response of the female-derived hRMCs.

2. Results

2.1Comparison of LacCers and N-linked glycosylation in urine of lupus nephritis patients compared to healthy controls, and between sexes

The study population comparing lupus nephritis (LN) patients to healthy controls (HCs) included 20 subjects in each group with equal sex distribution (50% female for each group). LN patients and HCs were similar in age but a larger proportion of LN

patients were black relative to HCs. The eGFR and urine creatinine levels were similar in the two groups. Significant differences between LN patients and HCs were observed for UPCr, C3 complement, and C4 complement. Participant demographics by disease status are show in Table 1. We previously demonstrated that levels of LacCers in the urine of LN patients were significantly higher compared to lupus patients without nephritis and compared to HCs [7]. Similarly, in this study cohort, the levels of the major chain lengths of LacCer (C16, C22, C24:1 and C24) as well as the total of all LacCer chain lengths were significantly higher in LN patients compared to HCs (Fig. 1A). To determine if LacCers levels differed based on biologic sex, we compared LacCers levels in the two groups. Although the differences did not reach statistical significance in this small cohort, the urine LacCers levels tended to be higher in females compared to males in the HC group (Fig. 1B). This trend was not observed in the LN group. Thus, the relative increases in LN urine LacCers (C16, C24:1, C24, and total) compared to HC urine was approximately two-fold higher in LN males than in LN females (Fig. 1C). This suggests that while both sexes with LN experience increases in urine LacCers, males may have a larger increase than females.

Table 1. Demographics and clinic measures of healthy controls (HC) and lupus nephritis (LN) patients.

	HC (N = 20)	LN (N = 20)	P
Sex, male, n (%)	10 (50.0)	10 (50.0)	1.000
Age, mean (SD)	34.0 (10.3)	33.2 (11.2)	0.824
Race, n (%)			0.041
Black	10 (50.0)	16 (80.0)	
White	10 (50.0)	3 (15.0)	
Other	0 (0.00)	1 (5.0)	
Estimated Glomerular Filtration Rate, mean (SD)	103.9 (23.9)	93.0 (51.9)	0.455
Urine Creatinine, mg/ml (SD)	1.46 (1.14)	1.24 (0.82)	0.484
Urine Protein: Creatinine, median (IQR)	0.055 (0.033)	1.69 (3.48)	<0.001
Nephritis Class, n (%)			N/A
I	N/A	2 (10)	
II	N/A	2 (10)	
III, IV	N/A	8 (40)	
III+V, IV+V	N/A	3 (15)	
V	N/A	3 (15)	
No biopsy/missing	N/A	2 (10)	
SLEDAI, mean (SD)	N/A	11.85 (5.6)	N/A
C3 Complement, mean (SD)	151.0 (16.1)	85.8 (21.5)	<0.001
C4 Complement, mean (SD)	33.9 (11.5)	23.0 (8.18)	0.034

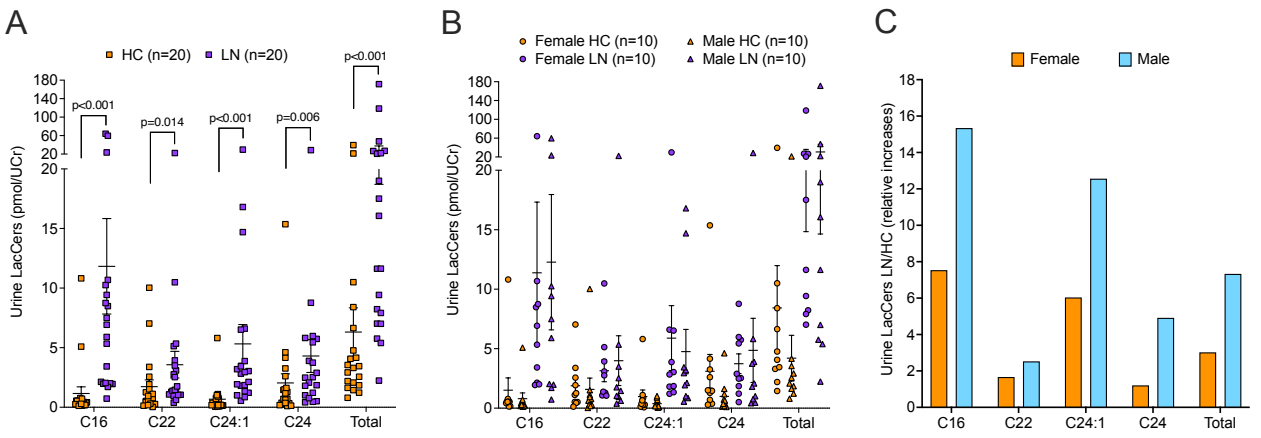

Figure 1. Urine LacCers levels were significantly higher in LN compared to HC. LacCer chain lengths of C14 to C26 were quantified in urine samples of healthy controls (HC) and lupus nephritis patients (LN), and included 10 females and 10 males in each group. A) Levels of the major LacCer chain lengths detected in the urine and the total of all LacCers chain lengths (Total) are shown in the graphs for all HC (n=20) compared to all LN (n=20) subjects. B) Levels of the major LacCer chain lengths by sex in the HC and LN groups. C) Ratio of LacCers levels in LN to HC by sex.

Ninety-six individual N-glycans (peaks) in urine were detectable in most of the samples, summarized in supplemental Table S2. Nine classes of glycans, which used the sum of the relative frequencies for those peaks in that class, were also considered: mannose, hybrid, biantennary, triantennary, tetrantennary, bisecting, fucosylated, sialylated, and sulfonated. Seven of the N-glycan classes differed significantly between LN patients and HCs after FDR correction (Fig. 2A). Of the 96 individual N-glycans detected, the relative abundance of 72 of the N-glycans differed significantly between LN patients and HCs after FDR correction. The top 10 significantly different individual N-glycans are presented in Fig. 2C. We also examined sex differences in the relative frequencies of the glycans in these data. The interaction between biologic sex and disease status was not significant in any of the statistical models. Thus, results for disease status are reported across males and females and results for biologic sex are reported across disease status. None of the N-glycan classes differed significantly by sex (Fig. 2B) and only three of the individual N-glycans (peaks 2361, 2339, and 2289, Fig. 2D) differed significantly by sex after FDR correction. There was a significant increase in the degalactosylated (and desialylated, peak 1485) glycan shown to be associated with IgG that exhibits a more pro-inflammatory function, and significant decreases in the biantennary galactosylated (peaks 1663 and 1809) and sialylated (peaks 2122 and 2435) glycans that are associated with a more anti-inflammatory IgG (Fig. 2E). Supplemental Fig. 1SA shows a heatmap of the 72 glycans found to be associated with disease status and supplementary Table S2 shows the mean difference and 95% confidence interval in the relative frequencies of all the N-glycan classes and individual peaks between LN patients and HCs, and between males and females.

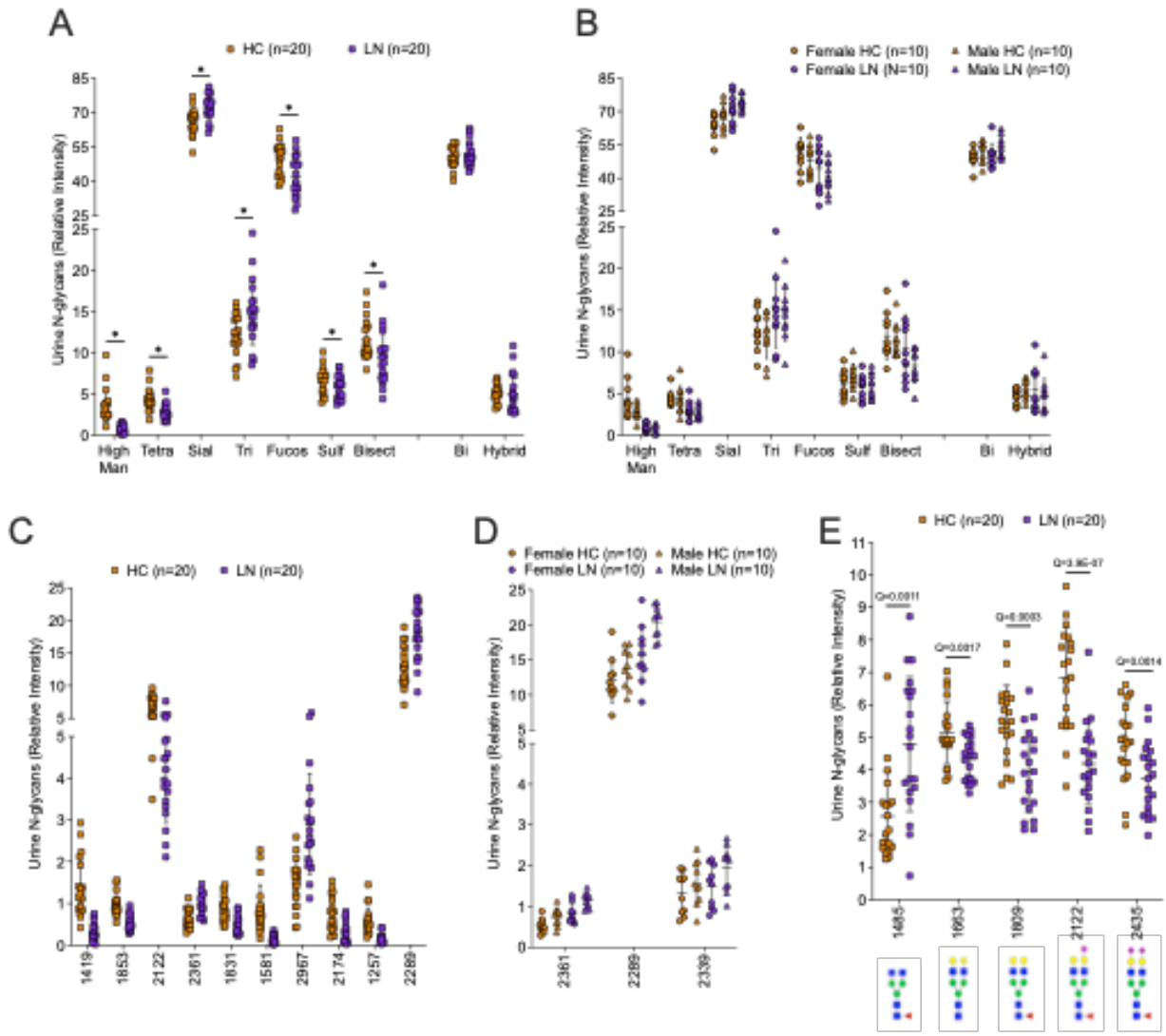

Figure 2. The urine N-glycome was significantly different in LN compared to HC, some differed by sex. N-glycans were quantified in the same urine samples as in Fig. 1 of 20 healthy controls (HC) and 20 lupus nephritis patients (LN), and included 10 females and 10 males in each group. N-glycan classes: high mannose (High Man), tetra-antennary (Tetra), sialylated (Sial), tri-antennary (Tri), fucosylated (Fucos), sulfated (Sulf), bisected (Bisect), bi-antennary (Bi), or Hybrid were compared in all HC vs all LN (A) or by sex in each group (B). Individual N-glycans were compared in all HC vs all LN (C) or by sex in each group (D). Of the 72 individual N-glycans that differed significantly between HC and LN, the top 10 are presented in (C). Only three N-glycans differed significantly between females and males (D). See Table S2 (Additional File 1) for a list of all urine N-glycans detected and the adjusted Q values, and Table S1 (Additional File 2) for N-glycans structure information.

We then examined if inclusion of the individual N-glycans in a model that included total urine LacCers level and biologic sex improved discrimination between LN patients and HCs for those N-glycans identified as differing between LN and HC. Models with one N-glycan added were compared to the baseline model for improvements in fit based on the likelihood ratio test. Of the 72 N-glycans identified to be associated with disease, 27 were significant in a model including total urine LacCers and biologic sex (although none retained significance after FDR correction). The primary metric for evaluating discrimination of cases was the AUC statistic. The baseline model including only urine total LacCers and biologic sex had an AUC (95% CI) of 0.87 (0.75, 0.99). Models including one

additional N-glycan from among those associated with SLE status improved the AUC relative to the baseline model by between 0 and 0.118 units (i.e. increasing the AUC from 0.870 to between 0.870-0.992). The AUCs, likelihood ratio test p-values (for comparison of a model including the N-glycan versus excluding the N-glycan), and difference in AUCs are shown in Table 2.

Table 2. AUCs, likelihood ratio test (LRT) p-values, and differences in AUC of adding urine N-glycans to a "null" model. The null model included only total urine LacCers and biologic sex. Urine LacCers was natural log transformed prior to fitting the models to meet statistical assumptions. *Inclusion of this glycan yielded perfect separation of the cases and controls.

	AIC	LRT p-value	AUC (95% CI)	D AUC (95% CI)
Null Model	43.12	0.0020	0.870 (0.748, 0.992)	
2669*	8	0.9970	1 (1, 1)	0.13 (0.008, 0.252)
1419*	8	0.9976	1 (1, 1)	0.13 (0.008, 0.252)
1581*	8	0.9987	1 (1, 1)	0.13 (0.008, 0.252)
Mannose*	8	0.9987	1 (1, 1)	0.13 (0.008, 0.252)
1485	33.92	0.0081	0.932 (0.859, 1.00)	0.062 (-0.031, 0.156)
1853	30.1	0.0081	0.945 (0.866, 1.00)	0.075 (-0.025, 0.175)
2174	32.47	0.0085	0.943 (0.876, 1.00)	0.073 (-0.036, 0.181)
1866	31.85	0.0095	0.948 (0.88, 1.00)	0.078 (-0.023, 0.178)
2122	33.54	0.0107	0.943 (0.872, 1.00)	0.073 (-0.047, 0.192)
2158	28.28	0.0108	0.958 (0.903, 1.00)	0.088 (-0.023, 0.198)
1996	31.62	0.0111	0.948 (0.887, 1.00)	0.078 (-0.026, 0.181)
2377	25.13	0.0119	0.970(0.923, 1.00)	0.100 (-0.019, 0.219)
2361	34	0.0124	0.938 (0.860, 1.00)	0.068 (-0.018, 0.153)
Tetraantennary	33.13	0.0130	0.938 (0.860, 1.00)	0.068 (-0.054, 0.189)
2012	34.74	0.0131	0.917 (0.830, 1.00)	0.047 (-0.062, 0.157)
2967	31.54	0.0159	0.943 (0.861, 1.00)	0.073 (-0.042, 0.187)
2289	36.13	0.0170	0.915 (0.828, 1.00)	0.045 (-0.059, 0.149)
Sialylation	35.54	0.0182	0.948 (0.874, 1.00)	0.078 (-0.018, 0.173)
1831	34.34	0.0212	0.932 (0.846, 1.00)	0.062 (-0.014, 0.139)
2056	37.2	0.0225	0.915 (0.829, 1.00)	0.045 (-0.025, 0.115)
1704	33.24	0.0242	0.955 (0.886, 1.00)	0.085 (-0.011, 0.181)
1809	37.7	0.0250	0.917 (0.833, 1.00)	0.047 (-0.036, 0.131)
3770	37.38	0.0264	0.902 (0.805, 1.00)	0.032 (-0.062, 0.127)
2632	38.17	0.0289	0.907 (0.813, 1.00)	0.037 (-0.035, 0.110)
2487	38.8	0.0311	0.897 (0.806, 0.989)	0.027 (-0.065, 0.120)
2267	37.86	0.0314	0.915 (0.830, 1.00)	0.045 (-0.038, 0.128)
1257	20.36	0.0346	0.985 (0.959, 1.00)	0.115 (-0.005, 0.235)
2245	38.88	0.0363	0.902 (0.807, 0.998)	0.032 (-0.034, 0.099)
2852	38.39	0.0367	0.912 (0.823, 1.00)	0.042 (-0.068, 0.153)
1079	39.5	0.0372	0.900 (0.793, 1.00)	0.030 (-0.067, 0.127)
2221	39.48	0.0389	0.902 (0.807, 0.998)	0.032 (-0.047, 0.112)
2638	38.44	0.0406	0.910 (0.809, 1.00)	0.040 (-0.040, 0.120)

3333	38.94	0.0412	0.897 (0.800, 0.995)	0.027 (-0.070, 0.125)
2287	38.33	0.0445	0.902 (0.808, 0.997)	0.032 (-0.040, 0.105)
1663	39.57	0.0477	0.885 (0.774, 0.996)	0.015 (-0.032, 0.062)
1891	19.61	0.0557	0.988 (0.965, 1.00)	0.118 (-0.001, 0.236)
1743	19.12	0.0567	0.985 (0.957, 1.00)	0.115 (0.005, 0.225)
2945	40.2	0.0586	0.897 (0.801, 0.994)	0.027 (-0.032, 0.087)
1905	21.5	0.0604	0.983 (0.954, 1.00)	0.113 (0.001, 0.224)
1444	37.27	0.0657	0.920 (0.831, 1.00)	0.05 (-0.038, 0.138)
2465	41.09	0.0714	0.890 (0.792, 0.988)	0.02 (-0.053, 0.093)
1960	41.41	0.0741	0.887 (0.774, 1.00)	0.017 (-0.038, 0.073)
2523	37.65	0.0813	0.907 (0.809, 1.00)	0.037 (-0.024, 0.099)
3144	40.32	0.0826	0.897 (0.800, 0.995)	0.027 (-0.05, 0.105)
1647	41.44	0.0864	0.895 (0.798, 0.992)	0.025 (-0.04, 0.09)
3646	41.69	0.0948	0.887 (0.787, 0.988)	0.017 (-0.043, 0.078)
2654	41.54	0.1038	0.887 (0.779, 0.996)	0.017 (-0.059, 0.094)
1954	42.03	0.1056	0.892 (0.790, 0.995)	0.022 (-0.036, 0.081)
2610	41.41	0.1125	0.885 (0.777, 0.993)	0.015 (-0.038, 0.068)
2028	42.3	0.1158	0.893 (0.778, 1.00)	0.023 (-0.024, 0.069)
sulfation	42.4	0.1233	0.900 (0.793, 1.00)	0.03 (-0.016, 0.076)
2163	42.2	0.1234	0.882 (0.774, 0.991)	0.012 (-0.062, 0.087)
2923	42.01	0.1353	0.880 (0.774, 0.986)	0.01 (-0.052, 0.072)
1814	42.61	0.1363	0.885 (0.786, 0.984)	0.015 (-0.061, 0.091)
2304	42.78	0.1480	0.887 (0.784, 0.991)	0.017 (-0.057, 0.092)
3092	42.8	0.1579	0.873 (0.754, 0.991)	0.003 (-0.034, 0.039)
1850	42.93	0.1609	0.88 (0.771, 0.989)	0.01 (-0.059, 0.079)
3193	42.38	0.1649	0.885 (0.778, 0.992)	0.015 (-0.042, 0.072)
3113	42.56	0.1651	0.882 (0.769, 0.996)	0.012 (-0.017, 0.042)
3004	42.8	0.1815	0.878 (0.760, 0.995)	0.008 (-0.03, 0.045)
2435	43.22	0.1838	0.875 (0.769, 0.981)	0.005 (-0.06, 0.07)
3093	43.2	0.1933	0.865 (0.740, 0.990)	-0.005 (-0.039, 0.029)
2393	43.16	0.2030	0.880 (0.769, 0.991)	0.010 (-0.042, 0.062)
fucosylation	43.35	0.2073	0.885 (0.780, 0.990)	0.015 (-0.044, 0.074)
2100	43.33	0.2168	0.870 (0.754, 0.986)	0.00 (-0.048, 0.048)
3384	43.18	0.2193	0.873 (0.755, 0.99)	0.003 (-0.044, 0.049)
triantennary	43.7	0.2642	0.870 (0.747, 0.993)	0.00 (-0.052, 0.052)
bisect	43.82	0.2776	0.882 (0.774, 0.991)	0.012 (-0.045, 0.07)
2319	43.58	0.2828	0.882 (0.772, 0.993)	0.012 (-0.036, 0.061)
1875	44.09	0.3257	0.882 (0.769, 0.996)	0.012 (-0.017, 0.042)
2383	44.55	0.4570	0.882 (0.767, 0.998)	0.012 (-0.019, 0.044)
2413	44.69	0.5224	0.863 (0.739, 0.986)	-0.007 (-0.041, 0.026)
1773	44.75	0.5600	0.875 (0.756, 0.994)	0.005 (-0.016, 0.026)
1611	44.83	0.5933	0.877 (0.762, 0.993)	0.007 (-0.02, 0.035)

2594	44.87	0.6229	0.877 (0.759, 0.996)	0.007 (-0.019, 0.034)
2339	44.89	0.6393	0.875 (0.752, 0.998)	0.005 (-0.019, 0.029)
2341	44.97	0.7073	0.875 (0.758, 0.992)	0.005 (-0.019, 0.029)
2391	45.1	0.9027	0.873 (0.750, 0.995)	0.003 (-0.004, 0.009)

2.2 Comparison of LacCers and N-linked glycosylation in serum of lupus nephritis patients compared to healthy controls, and between sexes

Serum samples were analyzed from the same LN patients and HCs from whom urine samples were analyzed above. As we reported previously in a different cohort of subjects [7], we did not observe a significant difference in C16 LacCer levels between LN patients and HCs in this cohort, nor did we observe a difference in the levels of total LacCers (Fig. 3A). However, we did observe significant differences in C22 and C24 LacCers between LN patients and HCs. As in the urine LacCers analyses, we did not observe any differences in serum LacCers based on biological sex regardless of disease status (Fig. 3B).

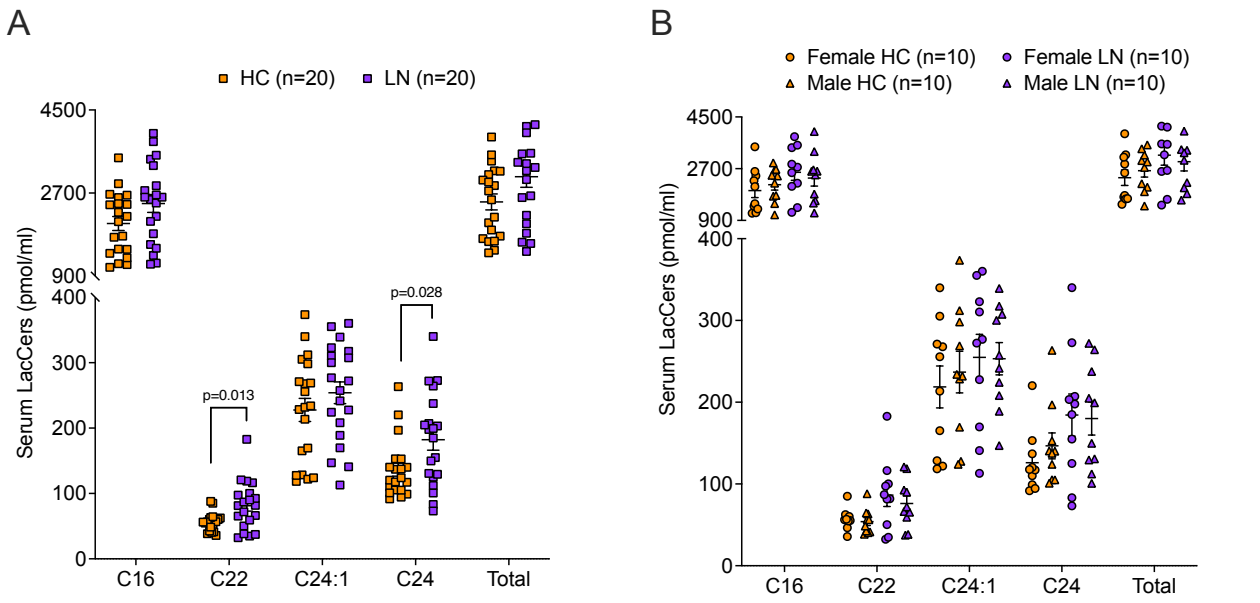

Figure 3. Two serum LacCer species were significantly higher in serum from LN compared to HC. LacCer chain lengths of C14 to C26 were quantified in serum samples of the same HC and LN subjects as in Fig. 1 (10 females and 10 males in each group). A) Levels of the major LacCer chain lengths detected in the serum and the total of all LacCers chain lengths (Total) are shown in the graphs for all HC (n=20) compared to all LN (n=20) subjects. B) Levels of the major LacCer chain lengths by sex in the HC and LN groups.

Seventy individual N-glycans (peaks) were detectable in serum in most of the samples. Five N-glycan classes differed significantly between LN patients and HCs after FDR correction (Fig. 4A). Of the 70 detected individual N-glycans, the relative abundance of 21 of the N-glycans groups differed significantly between LN patients and HCs after FDR correction. The top 10 significantly different individual N-glycans are shown in Fig. 4B. We also examined sex differences in the relative frequencies of the N-glycans in these data; however none of the N-glycan classes (Fig. 4C) or individual N-glycans differed significantly by sex after FDR correction. Only one of the IgG-associated N-glycan peaks, 1809 (desialylated containing a core fucose), in the serum was highly

significantly different between the two groups with it being decreased in the LN group (Fig. 4D). The mono-sialylated form was also decreased with the difference only just significant at Q=0.0449. Supplementary Fig. S1B shows a heatmap of the 26 serum N-glycans (21 individual and 5 classes) associated with disease status. Supplementary Table

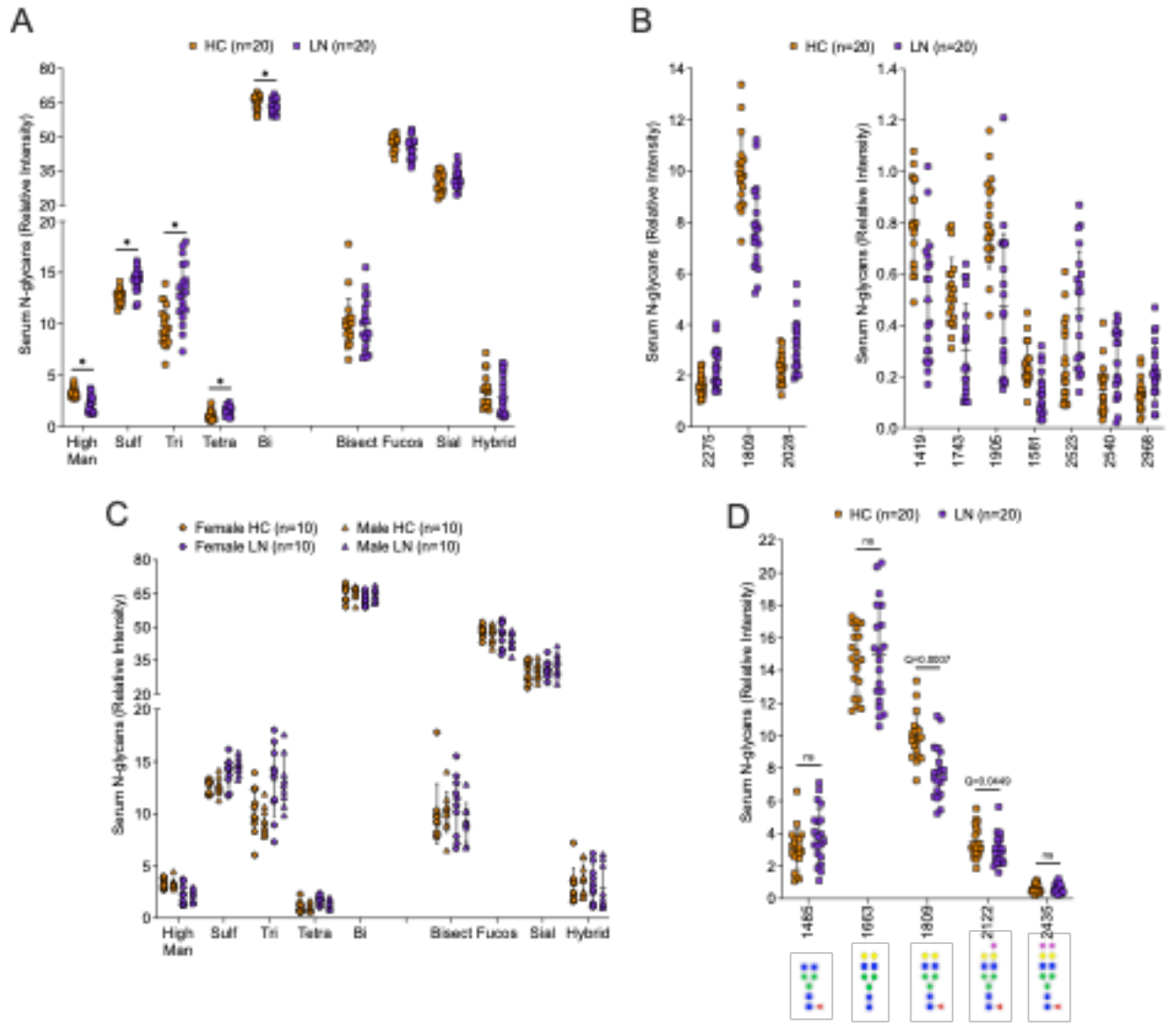

Figure 4. The serum N-glycome was significantly different in LN compared to HC. N-glycans were quantified in the same serum samples as in Fig. 3 of 20 healthy controls (HC) and 20 lupus nephritis patients (LN) and included 10 females and 10 males in each group. N-glycan classes: high mannose (High Man), tetra-antennary (Tetra), sialylated (Sial), tri-antennary (Tri), fucosylated (Fucos), sulfated (Sulf), bisected (Bisect), bi-antennary (Bi), or Hybrid were compared in all HC vs all LN (A) or by sex in each group (C) *Q<0.05 (see Table S3 for calculated, adjusted Q-values). B) Individual N-glycans were compared in all HC vs all LN and the top 10 of the 21 individual N-glycans that differed significantly between HC and LN are presented. See Table S3 (Additional File 1) for a list of all serum N-glycans detected and the calculated, adjusted Q-values, and Table S1 (Additional File 2) for N-glycans structures.

S3 shows the mean difference and 95% confidence interval in the relative frequencies of the different N-glycan classes or individual N-glycans between LN patients and HCs, and between males and females.

We then examined if inclusion of the individual serum N-glycans in a model including total urine LacCers level and biologic sex improved discrimination between LN and HC for those N-glycans identified as differing between LN patients and HCs.

Models with one N-glycan added were compared to the baseline model for improvements in fit base on the likelihood ratio test. Of the 21 individual or 5 classes of N-glycans associated with SLE status, 20 were significant in a model including total urine LacCers and biologic sex (although none retained significance after FDR correction). The baseline model including only urine total LacCers and biologic sex had an AUC (95% CI) of 0.87 (0.75, 0.99). Models including one additional N-glycan from among those identified to be associated with LN status improved the AUC relative to the baseline model by between 0.005 and 0.085 units. The AUCs, likelihood ratio test p-values (for comparison of a model including the serum N-glycan versus excluding the N-glycan), and difference in AUCs are shown in Table 3.

Table 3. AUCs, likelihood ratio test (LRT) p-values, and differences in AUC of adding serum N-glycans to a "null" model (same null model as in Table 2).

	AIC	LRT p-value	AUC (95% CI)	D AUC
Null Model	43.12	0.002	0.870 (0.748, 0.992)	
mannose	29.08	0.012	0.955 (0.900, 1.00)	0.085 (-0.016, 0.186)
1743	29.71	0.013	0.953 (0.897, 1.00)	0.083 (-0.013, 0.178)
1581	30.74	0.008	0.950 (0.887, 1.00)	0.080 (-0.024, 0.184)
sulfation	32.53	0.006	0.945 (0.875, 1.00)	0.075 (-0.031, 0.181)
1419	32.62	0.006	0.943 (0.876, 1.00)	0.073 (-0.030, 0.175)
triantennary	32.94	0.013	0.935 (0.867, 1.00)	0.065 (-0.041, 0.171)
1809	33.3	0.009	0.938 (0.868, 1.00)	0.068 (-0.039, 0.174)
2275	33.33	0.016	0.927 (0.852, 1.00)	0.057 (-0.041, 0.156)
2540.1	33.69	0.007	0.935 (0.864, 1.00)	0.065 (-0.023, 0.153)
2028	34.96	0.02	0.917 (0.834, 1.00)	0.047 (-0.052, 0.147)
1444	35.96	0.028	0.917 (0.834, 1.00)	0.047 (-0.044, 0.139)
1905	36.12	0.015	0.935 (0.855, 1.00)	0.065 (-0.009, 0.139)
1136	36.24	0.019	0.910 (0.823, 0.997)	0.040 (-0.054, 0.134)
tetraantennary	36.79	0.015	0.915 (0.830, 1.00)	0.045 (-0.049, 0.139)
1257	36.87	0.014	0.920 (0.831, 1.00)	0.050 (-0.044, 0.144)
2319	37.69	0.026	0.900 (0.809, 0.991)	0.030 (-0.072, 0.132)
2122	38.32	0.024	0.910 (0.826, 0.994)	0.040 (-0.053, 0.133)
2633.1	39.17	0.043	0.900 (0.809, 0.991)	0.030 (-0.060, 0.120)
2523.1	39.2	0.036	0.895 (0.799, 0.991)	0.025 (-0.059, 0.109)
2231	40.01	0.053	0.897 (0.803, 0.992)	0.027 (-0.050, 0.105)
2393	40.16	0.04	0.905 (0.813, 0.997)	0.035 (-0.045, 0.115)
2341	40.65	0.066	0.895 (0.799, 0.991)	0.025 (-0.050, 0.100)
biantennary	40.83	0.053	0.897 (0.788, 1.00)	0.027 (-0.036, 0.091)
1647	41.87	0.091	0.890 (0.780, 1.00)	0.020 (-0.037, 0.077)
2968.1	42.67	0.15	0.880 (0.774, 0.986)	0.010 (-0.059, 0.079)
2655.1	42.75	0.158	0.875 (0.767, 0.983)	0.005 (-0.071, 0.081)

2.3 Influence of disease and biologic sex in the response of mesangial cells to human sera

The above results showed significant differences in the levels of 21 different N-glycans between LN and HC serum. To determine if renal cells would exhibit differential responses to these sera, primary human renal mesangial cells (hRMCs) were used for the following studies. A preliminary experiment was performed to identify cytokines that may be differentially secreted in response to LN compared to HC sera (see Supplemental methods). We first measured the release of IL-6 and MCP-1 in response to 10% sera collected from 12 HCs, 12 LN patients with active disease, and 12 LN patients with inactive disease (not the same subjects included the LacCers and N-glycan analyses) based on our prior studies in mouse [18,19]. No differences across the three groups were observed in IL-6 or MCP-1 release (Fig. S2A). The media from this experiment was then pooled to generate two samples per group and a cytokine array screened to identify cytokines that may be differentially released in response to HC vs LN Active vs LN Inactive sera. Results from this array (Fig. S2B) suggested that higher levels of CCL5 and CXCL5 were released in response to LN Active sera compared to LN Inactive or HC sera.

To evaluate hRMC response to the sera from the HC subjects and LN patients analyzed in Figs. 3 and 4, release of CCL5 and CXCL5 was measured after incubation with 5% serum from each subject. For these studies, we also examined if responses were impacted by the biologic sex from which the hRMCs were derived. No significant

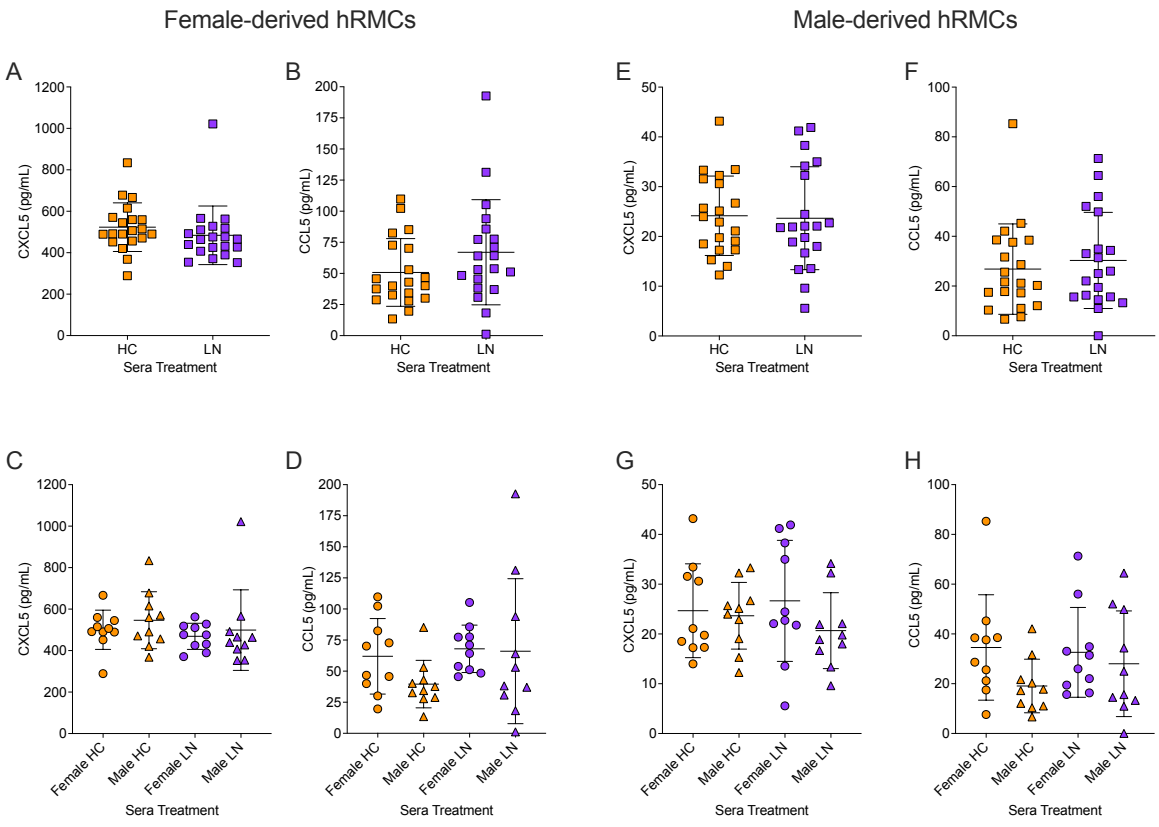

Figure 5. Cytokine release was higher in female-derived compared to male-derived hRMCs in response to sera. hRMCs were treated with 5% serum from 10 female or 10 male HC or from 10 female or 10 male LN subjects. A-D: female-derived hRMCs; and E-H: male-derived hRMCs. A, C, E, G: CXCL5; and B, D, F, H: CCL5 levels in media of sera-treated hRMCs. A, B, E, F: compares HC sera-treated to LN sera-treated (both female- and male-derived sera). C, D, G, H: compares female- and male-derived sera for each group (HC vs LN). Statistical analyses were performed using unpaired t-test for comparing HC vs LN or one-way Anova for comparing female vs male across the two groups. No significant differences were observed after correcting for multiple comparisons.

differences were observed between HC and LN sera treatments in the levels of CXCL5 (Fig. 5A) or CCL5 (Fig. 5B) released from the female-derived hRMCs. No significant differences in the release of CXCL5 (Fig. 5C) were present in response to sera with respect to biologic sex. However, a trend towards higher levels of CCL5 released in response to female HC compared to male HC sera was observed but not in response to female LN vs male LN sera (Fig. 5D). Similar results were obtained in the male-derived hRMCs (Fig. 5E-H), including a trend towards release of higher levels of CCL5 in response to female HC vs male HC sera (Fig. 5G). Interestingly, we observed that the levels of CXCL5 and CCL5 released were ~20-fold and ~2-fold higher, respectively, from the female-derived hRMCs (Fig. 5A-C) compared to the male-derived hRMCs (Fig. 5E-H). These differences were significant ($p<0.001$ for both CXCL5 and CCL5). These results suggest that the female-derived cells are pre-disposed to be hyper responsive to stimuli than the male-derived cells.

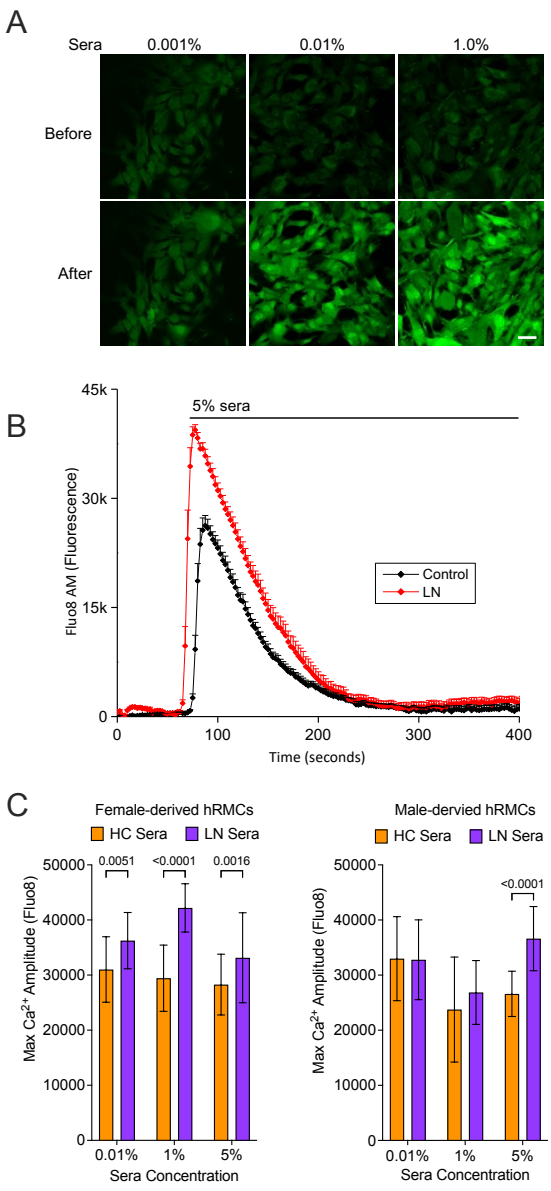

Figure 6. LN sera elicited a higher intracellular Ca^{2+} flux compared to HC sera in hRMCs. Sera was pooled from 10 female or 10 male HC (Control) or from 10 female or 10 male LN subjects and used to stimulate human primary hRMCs. Female-derived hRMCs were treated with HC or LN sera from females. Male-derived hRMCs were treated with HC or LN sera from males. A) Confocal fluorescent images of intracellular Ca^{2+} (Fluo-8 AM) in female hRMCs before and after addition of 0.01%, 0.01%, or 1% female HC sera. Scale bar 50 μm. B) Example of intracellular Ca^{2+} flux in response to acute application of 5% LN or HC sera in male hRMCs. C) Statistical analyses of maximal amplitude of intracellular Ca^{2+} flux in response to acute sera application.

To further assess the response of hRMCs to LN versus HC sera and the effect of biological sex of the cells, we measured intracellular calcium $[Ca^{2+}]_i$ flux in response to acute sera applications. Calcium transients in female- or male-derived hRMCs were observed in response to pooled same-sex HC or LN serum. The transient Ca^{2+} response was detected in the range of serum concentrations 0.001%, 0.01%, 0.1%, 1%, or 5% in 2 mM Ca^{2+} extracellular solution. The lowest

concentration of sera 0.001% promotes Ca^{2+} transients with the amplitude around 20% from the saturated values reached at the concentration of 0.01% sera for all groups. Fig. 6A illustrates representative confocal fluorescent (Fluo 8 AM) images of intracellular Ca^{2+} levels before and after acute application of 0.001%, 0.01%, or 1% sera. Examples of intracellular Ca^{2+} flux in response to acute application of 5% LN or HC sera in male hRMCs are shown in the Fig. 6B. Female-derived hRMCs exhibited significantly higher intracellular Ca^{2+} flux in response to LN compared to HC sera in all range of concentrations (Fig. 6C), and male-derived cells show similar pattern only at highest tested sera concentrations (5%). This data together with the results presented in Fig. 5, suggest that the female-derived hRMCs are more sensitive and respond more robustly to serum stimulation than the male-derived hRMCs.

2.4 Differences in LacCers and HexCers levels and the N-glycome in female-derived and male-derived hRMCs

We reported previously that increasing LacCers along with another glycosphingolipid, glucosylceramides (GlcCers), resulted in increased message levels of several cytokines in an immortalized mouse mesangial cell line [19]. We also showed that LacCers and GlcCers (or hexosylceramides, a combination of GlcCers and galactosylceramides) are increased in the renal cortex of lupus prone mice with nephritis [7], and that lupus patients with nephritis that did not respond to therapy had significantly higher levels of LacCers and HexCers prior to beginning treatment [20]. Thus, we measured LacCers and hexosylceramides (HexCers) in our female- and male-derived hRMCs. The levels of both LacCers (Fig. 7A) and HexCers (Fig. 7B) are higher in the female-derived compared to the male-derived hRMCs prior to any stimulation. The GSLs levels in the cells in Fig. 7 were measured in the serum-starved vehicle-treated wells from the experiments in Fig. 5. The observed differences in GlcCers and LacCers between the female- and male-derived hRMCs were verified in unmanipulated cells maintained in serum-containing medium at passages 5 and 6 (Fig. S3).

We also investigated differences in the N-glycome between the female-derived and male-derived hRMCs. The 17 most abundant N-glycans detected in both the female- and male-derived hRMCs are shown in Fig. 7C. The ten most abundant N-glycans comprised >60% of all N-glycans detected in these cells. In comparing the female- and male-derived cells, the type of N-glycans present and relative overall abundance of each of the N-glycans were similar. While several glycans showed trends of being more highly abundant (1743, 3486, 2853, 2393, and 1995) or less abundant (1809, 2122, and 2057) in the female-derived cells relative to the male-derived hRMCs, these differences were not as large as those observed for the GSLs in Fig. 7A-B. Thus, the analyses of GSLs and N-glycans in the hRMCs suggest that higher levels of LacCers and HexCers may contribute to a more robust response (higher cytokine release and increased intracellular Ca^{2+} flux) by the female-derived hRMCs following stimulation with sera.

3. Discussion

Given the ~9:1 female:male sex bias in lupus, most studies have focused largely on biologically female subjects (human and mouse studies). While men develop lupus less often than females, men were shown to have more severe disease and a higher risk of progressing to end stage renal disease [21,22]. However, the pathophysiologic mechanisms underlying sex differences are not fully understood. In this study, we determined that the significantly elevated LacCers and altered N-glycome in the urine can discriminate LN patients from HC subjects, and could serve as noninvasive definitive markers of LN. Alterations in the serum N-glycome may also be useful in discriminating LN from HC, but ultimately is less informative than the urine N-glycome in this respect. While we observed a few differences in the urine N-glycome in females compared to males, the

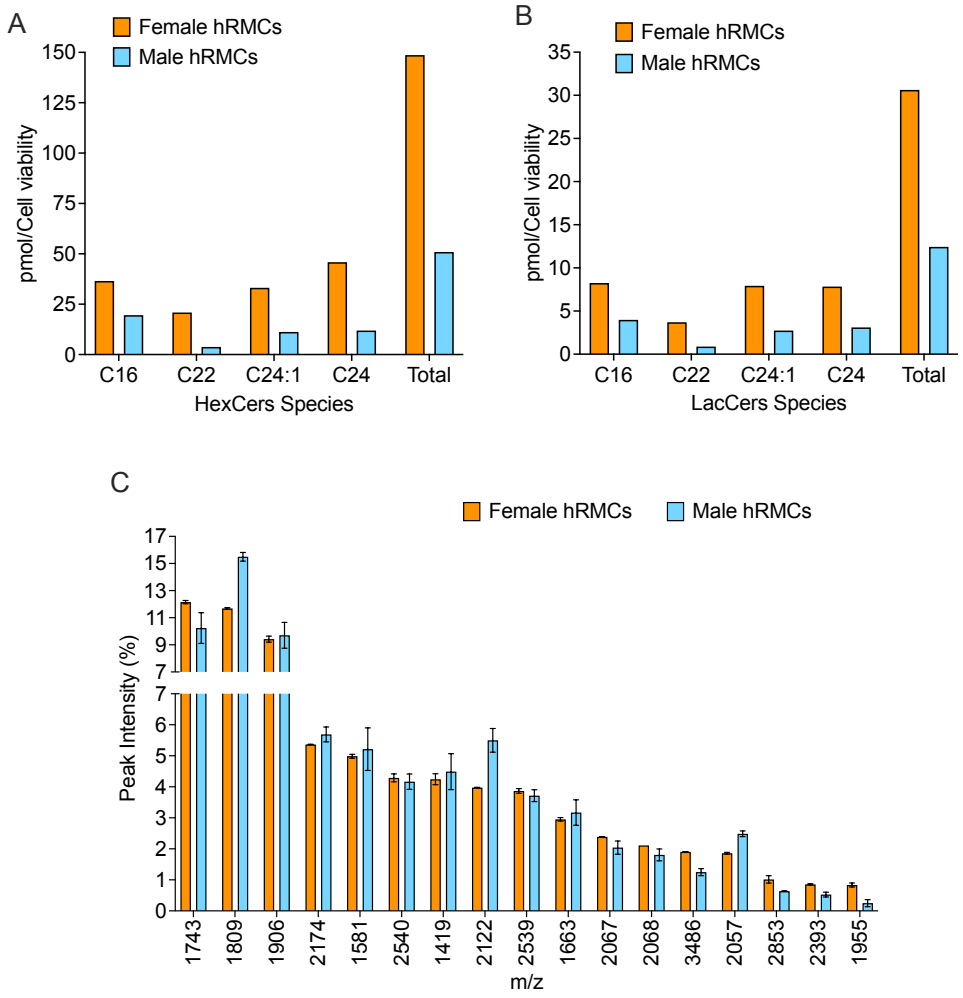

Figure 7. GSL levels were higher in female-derived compared to male-derived hRMCs. LacCers (A) and HexCers (B) were measured in female-derived and male-derived hRMCs and normalized to cell viability. The major chain lengths expressed in the hRMCs and the total of all chain lengths are shown in the graphs. The vehicle-treated female- or male-derived cells from the respective experiments presented in Figure 5 were scraped and combined to measure GLSs. GlcCers and LacCers levels were normalized to the cell viability determined prior to scraping. C) N-glycans were measured by MALDI-FTICR in female- or male-derived hRMCs plated in duplicate. Individual glycans are presented as the relative peak intensity of the total glycans measured in the individual wells. Means +SD shown on graphs. Statistical analyses were not performed for the GSL or glycan analyses since the measures were performed in hRMCs from only one female donor and one male donor.

levels of urine LacCers may be more informative with respect to sex differences. A recent study reported that men with SLE develop disease at a later age [23]. Our results suggest that LN males may experience a greater increase in LacCers than females when comparing the change in LacCers levels from HC to LN. LacCers levels measured in the urine likely are derived from the kidney [24]. Therefore, we hypothesize that lower levels of LacCers may be protective in males and contribute to the later disease onset, but once tolerance is broken and males begin to develop LN, the large increase in LacCers (or possibly glycosphingolipid metabolism in general) may contribute to worse disease.

This hypothesis is supported by our results in primary human mesangial cells (hRMCs) in which the response to stimuli seems to be more dependent on cellular differences rather than on the source of circulating stimuli. hRMCs released significant levels of CXCL5 and CCL5, and exhibited significant increases in intracellular Ca^{2+} in response to human sera. The female-derived hRMCs, which we demonstrated expressed higher levels of glycosphingolipids (GSLs) LacCers and HexCers, and released significantly more CCL5 and CXCL5 compared to the male-derived hRMCs in response to human serum (regardless of the source). At the lower concentrations of LN or HC sera the female-derived cells had a higher intracellular Ca^{2+} flux indicating that the female-derived hRMCs have an increased sensitivity to serum stimulation. GSLs modulate cellular functions such as proliferation, apoptosis, migration and signaling, including Ca^{2+} signaling [6], and defects in GSL metabolism are associated with a variety of human diseases. GSLs expressed on the cell surface form clusters and are widely believed to play roles in formation and stabilization of lipid domains ("lipid rafts") required to propagate extracellular signals. LacCers were shown to play a role in Lyn-mediated signaling in neutrophils [25-27] and MAPK signaling in cardiomyocytes [28], which leads to superoxide production, phagocytosis, migration or hypertrophy. In mesangial cells, elevated LacCers and HexCers due to hyperglycemia resulted in hypertrophy, extracellular matrix production, and fibrosis [29]. Our previous studies demonstrated that LN patients that failed to respond to treatment had significantly higher levels of HexCers and LacCers prior to beginning treatment [20]. Together, these observations suggest that renal GSL metabolism plays an important role in the scope (i.e. sensitivity or magnitude) of the initial renal response to stimuli and possibly resistance to therapeutic intervention. Thus, the elevated levels of LacCers and HexCers may poise the female-derived hRMCs to respond more robustly to external stimuli than the male-derived hRMCs.

Post-translational N-linked glycosylation plays an important role in the function of proteins, impacting a variety of cellular functions including discriminating self from non-self. Similar to GSLs, they can play key roles in mediating cell function. Modifications of N-glycosylation, or an altered N-glycome, is associated with several human diseases including lupus [15,30-32]. A recent study showed an abnormal N-glycome in renal biopsy sections of LN patients compared to renal biopsies from healthy tissue or from patients with other types of kidney conditions [16]. Here, we observed a significantly altered N-glycome in the urine of LN patients compared to HC subjects. This included seven of the nine classes and 75% of the individual N-glycans (72 of the 96) detected in urine. Overall, the urine glycan profiles were more informative in regard to disease status as compared with the N-glycans determined in the patient matched serum samples. The kidney biopsy study observed the largest difference in the abundance of mannose-enriched N-glycans, which was higher in the kidney of LN patients [16]. Conversely, we demonstrated a significant decrease in high mannose-containing N-glycans in the urine of LN patients in this study. The increase in mannose-containing N-glycans reported in the kidney [16] and the decrease we observed in high mannose-containing N-glycans in the urine may be due to differences in how the mannose-containing N-glycans were defined or grouped in the two analyses. Alternatively, differences in levels may be due to tissue versus secreted (into the urine). Future studies with matched urine and renal biopsies to compare levels within the same individuals using the same method of defining N-glycan classes is needed to address this question.

Age, sex, and body mass index (BMI) were associated with changes in the N-glycome [33-38]. Sex differences in the N-glycome reported in the literature are largely associated with IgG glycosylation. Pregnant women were reported to have higher levels of galactosylated and sialylated (anti-inflammatory) forms of IgG, which correlated with estrogen levels [33]. In a lupus study, estrogen was shown to alter IgG sialylation and induce an enzyme that adds sialic acid to N-glycans [39]. In our study, we observed that

individual N-glycans in the urine at peaks 2361, 2289, and 2339 differed by sex, with higher levels of all three observed in males compared to females regardless of disease status. However, none of these peaks are associated with IgG. Although we also demonstrated differences in the serum N-glycome between LN and HC, no sex differences were observed. Identifying the proteins from which the three urine N-glycans were derived that differed between sexes may lead to a better understanding of sex bias mechanisms in LN. Importantly, inclusion of four of the urine N-glycans associated with LN in a model including total urine LacCers and biologic sex improved the AUC to 1.0, providing perfect separation of LN from HC. Thus, measuring GSLs and N-glycans in the urine could serve as biomarkers of disease. Similarly, inclusion of the serum N-glycans improved the AUC in this model in distinguishing LN patients from HC subjects. Future studies using longitudinal serum samples to survey N-glycans in lupus patients who have not or have developed nephritis to determine if specific serum N-glycans can predict which lupus patients are likely to develop nephritis are of interest.

As mentioned above, many of the differences in glycosylation previously reported relate to IgG. Fc N-linked glycosylation influences the pathogenicity of IgG. Loss of sialic acid and galactose residues from the IgG N-glycome is associated with pro-inflammatory effector functions and autoimmune diseases [9]. Changes in glycosylation of serum IgG autoantibodies in lupus including decreased sialylation and galactosylation were reported previously and an altered IgG glycome was associated with disease status [14]. Here we observed significant differences in five N-glycans associated with IgG in the urine, and only two of those in the serum, of LN patients. In the urine, there was a significant increase in the degalactosylated (desialylated) glycans associated with proinflammatory IgG functions. This was coupled with corresponding significant decreases in the glycans associated with anti-inflammatory IgG functions. Interestingly, we did not observe a similar increase in the proinflammatory and decrease in anti-inflammatory IgG-associated glycans in the serum. Since the serum and urine samples were collected from the same patient at the same visit, it is possible that in LN, the more proinflammatory forms of IgG are deposited in the kidney (or other organs), reducing their levels in the circulation. Identification of the individual N-glycans present in the urine may lead to a better understanding of disease mechanisms in LN since most of the proteins present in the urine are likely derived from the kidney.

There were some limitations to this study. Except for the GSLs measures in Fig. 7A-B, our *in vitro* hRMC studies were performed using cells derived from one female donor and one male donor. Thus, it is possible that the differences observed are due to individual differences that are unrelated to the biologic sex of the donors. Additional analyses will need to be performed in a larger number of female and male donor-derived hRMCs to determine if there is a correlation between biologic sex and GSL metabolism (or N-glycome), and with pathological response. In addition, the female-derived hRMCs showed a significantly higher sensitivity in intracellular Ca^{2+} flux in response to LN sera, specifically at the low concentrations, which was similar to the higher release of CXCL5 and CCL5 in response to sera by the female-derived cells. Thus, future studies are needed to assess differences more globally (i.e. proliferation, apoptosis, or release of other cytokines, growth factors, or extracellular matrix proteins) in the effect of LN vs HC serum. Another limitation is the small cohort size. GSLs and the N-glycome can vary based on age or can be influenced by ethnicity or environmental factors. The HC and LN groups in this study were matched closely in average age, but the groups were too small to adjust for age, ethnicity, or external/environmental factors. Future studies with a larger cohort will be needed to confirm our observations.

4. Materials and Methods

4.1 Human samples and ethics statement

All results, except those in supplemental Fig. S2, analyzed stored urine and serum samples collected from the same subject at the same visit and included 20 healthy subjects (10 female and 10 male) and 20 lupus nephritis patients (10 female and 10 male). Lupus nephritis (LN) patients met the American College of Rheumatology classification for systemic lupus erythematosus (SLE) with nephritis and samples were collected during active disease. All except two LN patients had biopsy-confirmed nephritis. Healthy subjects (healthy controls, HC) did not have documented autoimmunity, renal disease, an active infection, or an ongoing pregnancy at the time of sample collection. Patient demographics and relevant clinic measures are provided in Table 1. Urine Protein:Creatinine ratio (UPCr), eGFR, C3, and C4 measures were missing for twelve of the HC subjects. The reported values in Table 1 are representative of the eight for whom measures were available. For analyses in supplemental Fig. S2, stored serum samples from 12 HC, 12 LN patients at the time of inactive disease, and 12 LN patients at the time of active disease were used to stimulate mesangial cells as described below. Inclusion and exclusion criteria for HC and LN subjects were the same as described above.

4.2 Cell culture

Two lots of primary human renal mesangial cells (hRMCs) were commercially obtained from ScienCell (Carlsbad, CA). Each lot was derived from one individual; 21-week gestation female (referred to as "female-derived") and one 22-week gestation male (referred to as male-derived). hRMCs were maintained on poly-l-lysine coated flasks in complete growth mesangial cell media (MCM) (1% penicillin/streptomycin and 1% mesangial cell growth supplement) that was supplemented with 2% FBS in a humidified, 5% CO_2 atmosphere at 37°C according to manufacturer's recommendations (ScienCell). Cells at passages 5 or 6 were used for experiments.

4.3 Lipid analyses

Glycosphingolipids hexosylceramides (HexCers) and lactosylceramides (LacCers) of individual chain lengths C16, C18, C18:1, C20, C20:1, C22, C22:1, C24, C24:1, C26, and C26:1 were quantified by the Lipidomics Core Facility at MUSC as we described previously [7,20,40]. The most highly expressed ("major") chain lengths in urine, serum, and hRMCs were C16, C22, C24, and C24:1 and quantified levels of these four chain lengths are provided on the graphs. The reported "total" HexCers or LacCers are the sum measures of all 11 chain lengths listed above. For urine, equivalent volumes of urine from each subject were provided and lipid measures were normalized to urine creatinine. Creatinine levels in all urine samples were measured in our laboratory in the same assay by the Jaffe picric acid method [41]. For serum, equivalent volumes of serum from each subject were provided to the core facility and lipids are presented as pmol of lipid per ml of serum. For the hRMCs, lipids were measured in cell pellets and are presented as pmol of lipids normalized to relative cell viability as measured by alamar blue just prior to collecting the cells.

4.4 N-glycan analyses

The N-glycan analysis of urine and serum was performed as previously described [42,43]. Serum was diluted 1:2 in 100 mM sodium bicarbonate pH 8.0 and 1 μL spotted on a Nexterion Slide H amine-reactive hydrogel-coated glass slide from Applied Microarrays (Tempe, AZ). Urine samples were buffer exchanged in phosphate buffered saline

and concentrated using a 0.5 ml Amicon 10,000 MW centrifugation tube prior to spotting [42]. After a 1-hour incubation, salts and lipids were removed using a Carnoy's solution (10% glacial acetic acid, 30% chloroform, 60% ethanol) wash. The samples were then sprayed with the enzyme peptide N-glycosidase F (PNGase F PRIME, N-Zymes Scientific, Doylestown,PA) and incubated for 2 hours to cleave N-glycans from the captured glycoproteins. Finally, an α-cyano-4-hydroxycinnamic acid (CHCA) matrix was sprayed onto the slides before performing MALDI-IMS using a Bruker 7T SolariX MALDI-FTICR mass spectrometer for serum and a Bruker MALDI-QTOF timsTOF fleX mass spectrometer for urine.

For cells, N-glycans were quantified as we previously described [18,44]. Briefly, female- or male-derived hRMCs cells were seeded at 6000 cells per well in duplicate wells on 8-well LabTekII chamber slides (Electron Microscopy Sciences, Hatfield, PA). Wells with no cells (media only) were used to determine background levels from media. Cells were washed with PBS, fixed for 30 min in 10% buffered formalin, washed and stored in PBS until analysis. N-glycans released by PNGase F digestion were detected by MALDI-FTICR as previously reported [18,44].

N-glycan peaks were analyzed using SCiLS Lab (v. 2021b) software (Bruker) as previously reported [42]. Mass spectra were normalized to total ion current. Peaks were selected for N-glycans based on theoretical and established mass values, and maximum mean values for each peak included in subsequent analyses. Background signal in the blank well was subtracted from each N-glycan measurement to obtain an absolute intensity. Relative intensities of N-glycans were calculated (absolute intensity divided by the intensity of all N-glycans detected in each sample). This accounted for protein concentration differences that could lead to higher signal intensities from sample to sample and allow for detection of low-abundance N-glycans. The sum of relative intensities of the individual N-glycans in a specific class was used to calculate each N-glycan classes (Bi-, Tri-, or Tetra-antennary, bisecting, and hybrid). In addition, each N-glycan was placed into a group based on the absence or presence of mannose, sulfate, sialic acid, or fucose. The sum of relative intensities in these classes/groups were then compared. Sialylated or sulfated N-glycans with multiple sodiated species were included together when comparing the intensities of individual N-glycans across samples. A cumulative peak and structure list of N-glycans used in the statistical analyses is provided in supplemental Table S1.

4.5 Cytokine release experiments

Female-derived or male-derived hRMCs described above were serum-starved for three hrs in serum-free complete MCM (without FBS supplementation) when ~80% confluent. Human sera were then added to a final concentration of 5%, incubated for three hrs, and refed with fresh serum-free complete MCM. Serum from a single individual was used to for all experiments. All treatments were performed in duplicate or triplicate. Media was collected from hRMCs following incubation with human sera. Cell viability was then measured using the Alamar Blue assay (Invitrogen/ThermoFisher) following the manufacturer's instructions. ELISA kits from Biolegend (San Diego, CA) were used to measure CCL5 (RANTES) or CXCL5 according to the manufacturer protocol. Relative cell viability (per well) with respect to untreated cells was used to normalize measured cytokine levels per well. For the individual serum analyses, replicates for each serum donor were averaged. The averages for each serum donor are shown on the graphs as individual points.

4.6 Intracellular Ca2+ analyses

The male- and female-derived hRMCs described above were used in confocal fluorescent experiments. The experiments were performed similarly to previously described protocol [45]. Briefly, cells were grown on glass-bottom dishes (Mattek, #0 glass) and loaded with fluorescent Ca^{2+} indicator Fluo-8 AM (AAT Bioquest). After loading, cells were rinsed and media was replaced with an extracellular solution containing in mM: 2 $CaCl_2$, 145 NaCl, 2 $MgCl_2$, 4.5 KCl, 10 HEPES, pH 7.4 adjusted by NaOH. Confocal imaging was performed using the Leica TCS SP5 laser scanning microscope equipped with an HCX Plan Apochromat 40x, 1.25 NA oil objective (Leica). Maximum amplitude of intracellular Ca^{2+} transient in individual hRMCs was obtained in response to the application of human sera from LN or HC patients in concentrations from 0.01 to 5 % (at least three separate experiments per group). The data were analyzed using ImageJ (NIH) and summarized in OriginPro software (OriginLab).

4.7 Statistical analyses

Descriptive statistics were determined for participant characteristics by LN status. Differences in patient characteristics for categorical variables were examined using Fisher's exact tests and for continuous or ordinal variables were examined using 2-sample t-tests or Wilcoxon rank sum tests as appropriate.

Comparisons of LacCers or N-glycans in urine and serum by disease status and by biologic sex were examined associations using a series of linear mixed models. Fixed effects in the models included disease status and biologic sex. We also considered the disease status by biologic sex interaction but only retained it if it was statistically significant. For the N-glycans, all models also included a random batch effect to control for correlation between samples run in the same batch. P-values for the associations between LacCers or N-glycans with disease status or with sex were adjusted using FDR to control for multiple testing. All FDR q-values < 0.05 are considered meaningful. We also evaluated if including individual N-glycans improved prediction of disease status in a model including total urine LacCers and biologic sex using the likelihood ratio test to determine if inclusion of glycans improved prediction and area under the receiver operating characteristics curve (AUC) to examine improvement in ability to discriminate between LN patients and HC subjects.

Differences in CXCL5 and CCL5 production between hRMCs treated with sera from healthy controls versus lupus nephritis were also examined. Additional factors considered included serum source (male or female donors) and cell line biologic sex. Differences between groups in CXCL5 or CCL5 expression were evaluated using a linear model approach. Models included main effects for disease status of the serum donor (HC vs LN), sex of the serum donor, and sex of the hRMC donor. Two-way interactions between cell type, derived sex, and serum sex were considered but were not significant and thus only main effects were considered. Differences by disease status, serum sex, and derived cell sex were estimated using linear contrasts. P-values where Bonferroni adjusted for the 3 pairwise comparisons. Model assumptions where checked graphically and transformations were considered as needed.

5. Conclusions

This study demonstrates that altered GSLs and N-glycosylation could serve as effective biomarkers of LN, particularly in urine, and that elevated cellular GSLs levels in the female-derived hRMCs were associated with a greater response (higher levels of cytokine secretion and intracellular Ca^{2+} flux) to human sera. GSLs and N-glycans also warrant further investigation as potential predictive biomarkers of LN and future studies are needed to determine if the elevated levels of GSLs in the female-derived cells are due to

sex differences or other individual donor differences. Elucidating the mechanisms by which GSLs and an altered N-glycome contribute to disease, and specifically the response of renal cells to external stimuli, could provide a better understanding of disease pathology.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: title; Table S1: title; Video S1: title.

Author Contributions: BW performed the statistical analyses and assisted in the interpretation of results; CB and BW performed the urine and serum N-glycan analyses; SM performed the mesangial cell experiments and ELISAs; MS, MF, and OP designed and performed the Ca^{2+} imaging experiments; HB performed the N-glycan analyses of the mesangial cells; JR assisted with the processing of urine and serum samples and measured creatinine in the urine samples; RD assisted with the design and performance of all N-glycan analyses and interpretation of results; TKN conceived and designed the study, assisted with the design and performance of experiments, interpretation of results. All coauthors contributed to the drafting and editing of the manuscript. BW, CB, and SM contributed equally to this study.

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Institutional Review Board Statement: The study was approved by the MUSC Institutional Review Board and/or by the Department of Defense Human Research Protection Office. All volunteers provided informed consent for the use of their samples in research.

Informed Consent Statement: All subjects were seen at the Medical University of South Carolina (MUSC) clinics and provided informed consent for their samples to be used for research. The studies were approved by the Institutional Review Board at MUSC. All samples were obtained from the Core Center for Clinical Research (CCCR) biorepository at MUSC and were provided in a coded manner.

Data Availability Statement: All data supporting the conclusions of this study are available upon request to the corresponding author.

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Supplemental Methods, Figures, Tables

Methods for supplemental Figure S2

For this experiment, media from female-derived hRMCs were seeded in a 96-well plate, serum-starved, and incubated with donor serum from each of 12 individuals from the following three groups of donors: HC, LN with inactive disease, LN with active disease. These sera were from a separate cohort from the sera described in the main text. Incubations were performed in triplicate for each donor. Media was collected from each well and used for the individual IL-6 and MCP-1 ELISAs and screening a cytokine array as follows. For IL-6 and MCP-1 ELISAs (BioLegend), media from each well was run on the ELISAs, triplicates for each individual were averaged and graphed as an independent point (Figure S1A). The remaining media was then pooled to collect sufficient volume to screen a cytokine array. Two pools for each group (replicate wells of 6 individuals per group) was obtained and used to screen a 71-cytokine array (Eve Technologies, Calgary, Canada). The two samples per group were averaged and the means +SD are presented. Cytokine levels were normalized to cell viability using the alamar blue assay.

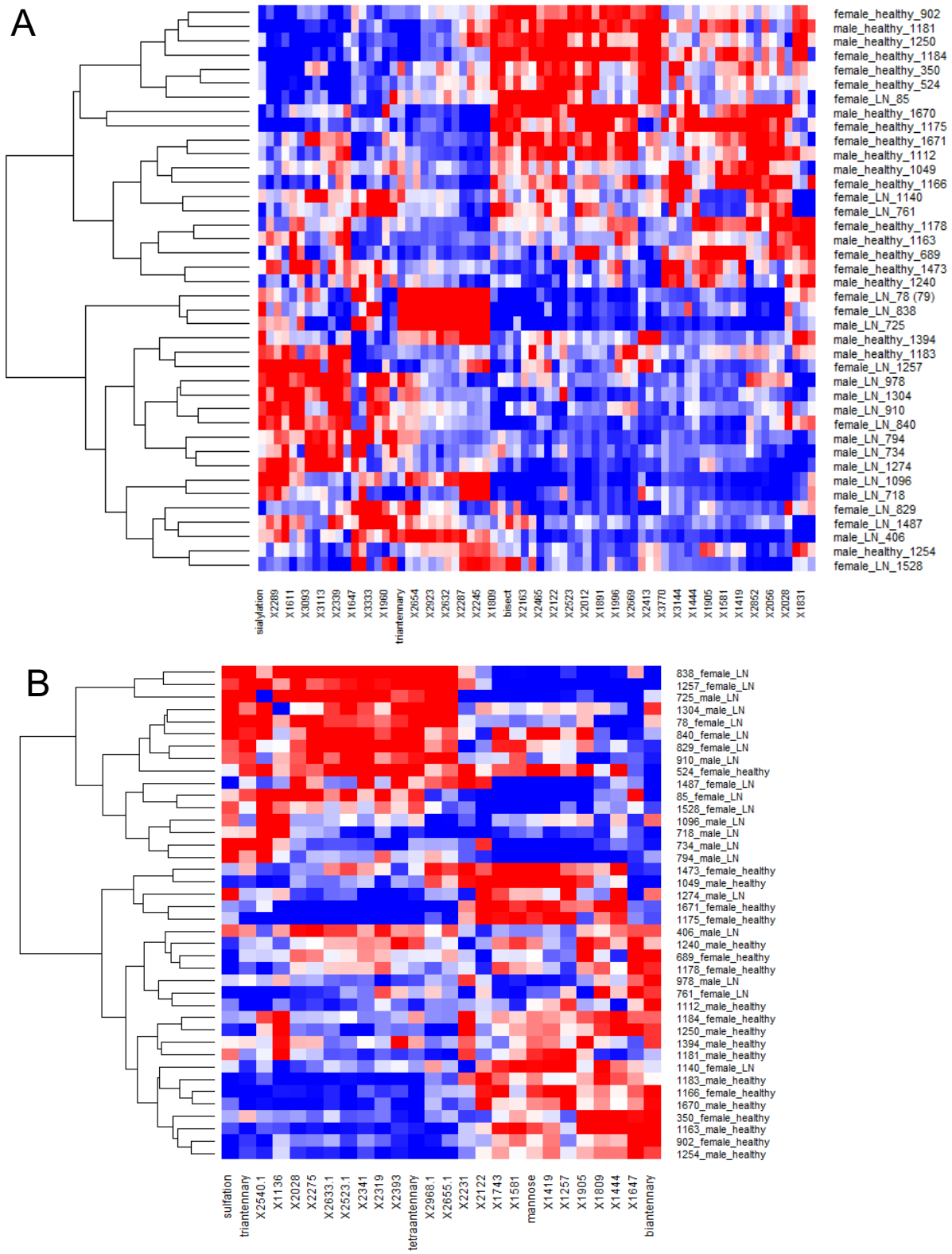


Figure S1. Heatmaps of N-glycans associated with disease status. Heatmaps of the 72 urine N-glycans (A) and 26 serum N-glycans (B) associated with disease status.

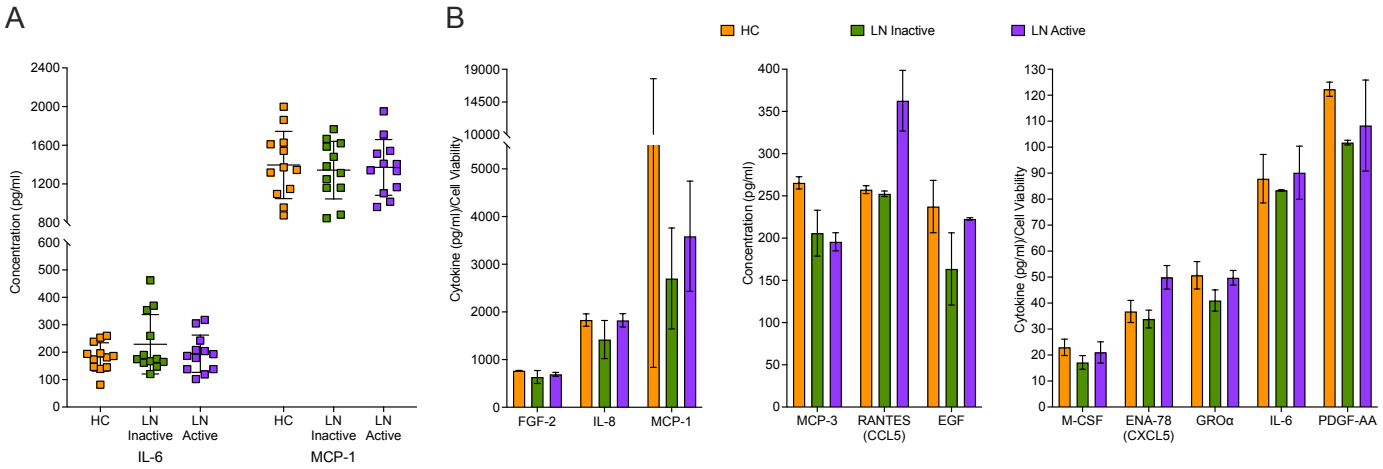


Figure S2. Cytokines released from female-derived primary human renal mesangial cells (hRMCs) in response to human sera. A) Female-derived hRMCs were stimulated with 10% human sera from 12 healthy control subjects (HC), 12 lupus nephritis patients with inactive disease (LN Inactive), or 12 lupus nephritis patients with active disease (LN Active). Treatments were performed in triplicate. The average of the triplicates for each subject is plotted on the graph. IL-6 and MCP-1 released into the media were quantified by ELISA and normalized to cell viability. B) Media was pooled from the cells treated in (A) within each group as described in the supplemental methods and used to screen an array containing 71 cytokines. Graphed cytokines include the most highly expressed cytokines. Levels were normalized to cell viability and levels measured in the media from untreated cells were subtracted. Since these analyses were from a single experiment, statistical analyses were not performed; however, CCL5 and CXCL5 showed trends of higher levels in the media of cells treated with LN Active sera compared to LN Inactive sera or HC sera. Thus, these two cytokines were chosen for analyses in subsequent experiments.

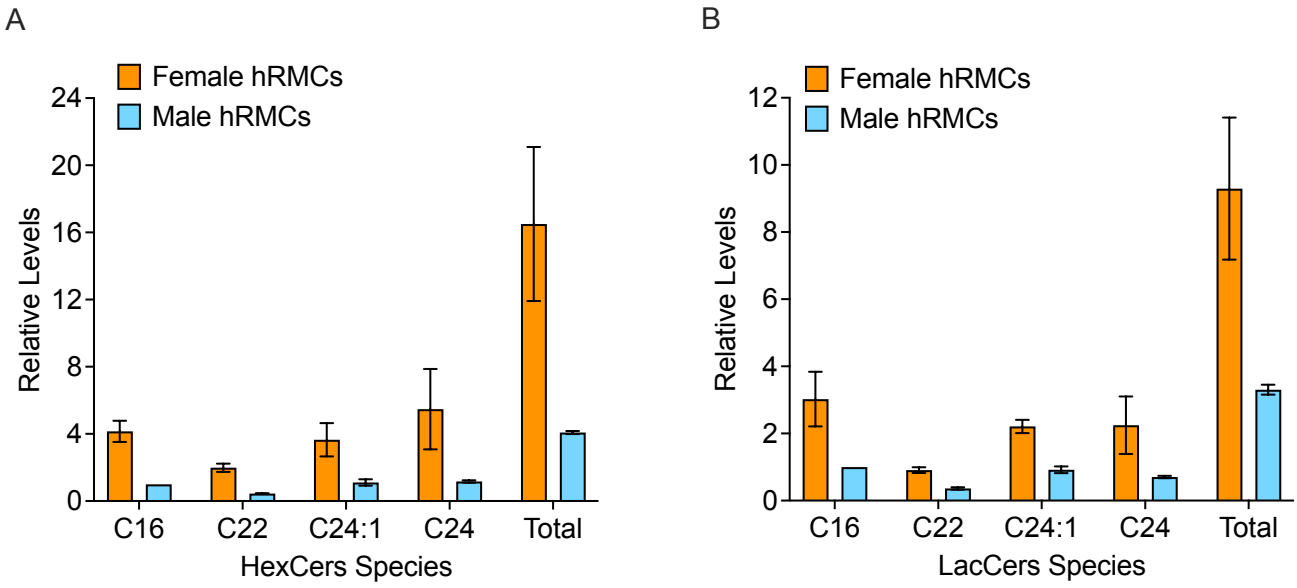
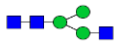
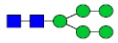
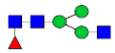
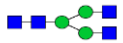
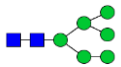
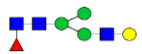
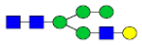
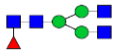
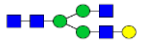
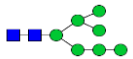
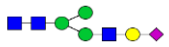
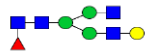
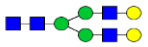
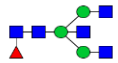
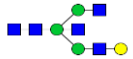
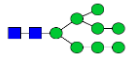
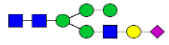
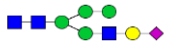
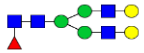
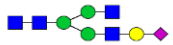
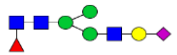
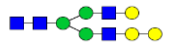
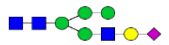
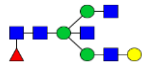
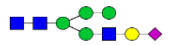
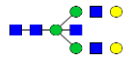
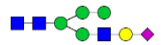
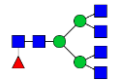
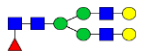


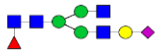
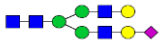
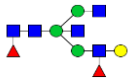
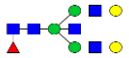
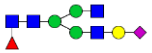
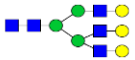
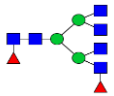
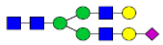
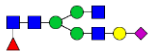
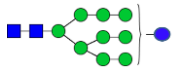
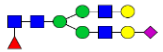
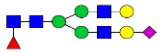
Figure S3. Female-derived hRMCs had higher levels of HexCers and LacCers. Female-derived and male-derived hRMCs were maintained in culture as indicated in the Methods section and one 10 mm plate of cells collected at passages 5 and 6 after measuring cell viability using alamar blue assay. HexCers (A) and LacCers (B) were measured for each passage and normalized to cell viability. Relative levels were calculated by setting the levels for HexCer or LacCer C16 to 1.0 for the male-derived cells and all other levels for both the male- and female-derived cells are relative to it within each passage. Normalized levels for each passage were averaged. Means +SD are presented. Statistical analyses were not performed since the measures were performed in hRMCs from only one female donor and one male donor.

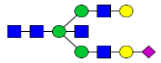
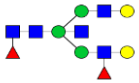
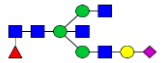
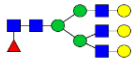
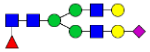
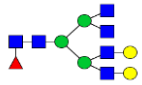
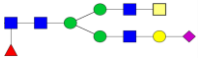
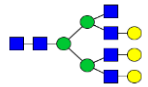
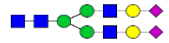
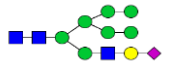
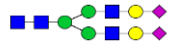
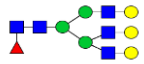
Supplementary Table 1. Cumulative peak list of detected N-glycans in serum, urine, and cells.

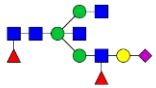
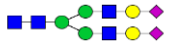
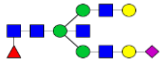
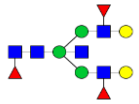
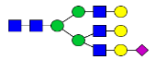
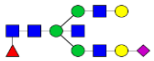
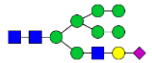
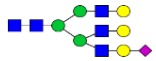
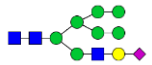
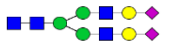
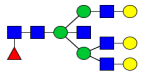
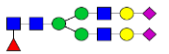
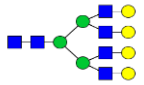
Theoretical Mass	Mass in Tables or Figures	Composition	Putative Structure	+SO4	+Na	Glycosylation Class
933.371	933	Hex3HexNAc2 + 1Na		0	1	
1079.375	1079	Hex3dHex1HexNAc2 + 1Na		0	1	Fuc
1095.370	1095	Hex4HexNAc2 + 1Na		0	1	Man
1136.396	1136	Hex3HexNAc3 + 1Na		0	1	
1257.423	1257	Hex5HexNAc2 + 1Na		0	1	Man
1282.454	1282	Hex3dHex1HexNAc3 + 1Na		0	1	Fuc
1298.449	1298	Hex4HexNAc3 + 1Na		0	1	
1339.476	1339	Hex3HexNAc4 + 1Na		0	1	Bia
1419.476	1419	Hex6HexNAc2 + 1Na		0	1	Man
1444.507	1444	Hex4dHex1HexNAc3 + 1Na		0	1	Fuc
1460.502	1460	Hex5HexNAc3 + 1Na		0	1	Hyb

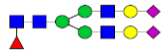
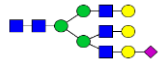
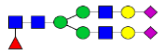
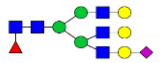
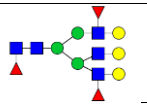
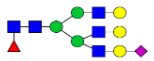
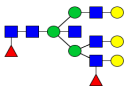
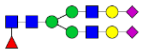
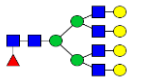
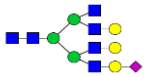
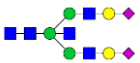
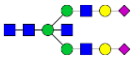
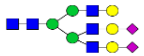
1485.534	1485	Hex3dHex1HexNAc4 + 1Na		0	1	Fuc, Bia
1501.529	1501	Hex4HexNAc4 + 1Na		0	1	Bia
1581.528	1581	Hex7HexNAc2 + 1Na		0	1	Man
1606.560	1606	Hex5dHex1HexNAc3 + 1Na		0	1	Fuc, Hyb
1611.527	1611	Hex4HexNAc3NeuAc1 + 2Na		0	2	Sia
1622.555	1622	Hex6HexNAc3 + 1Na		0	1	Hyb
1647.587	1647	Hex4dHex1HexNAc4 + 1Na		0	1	Fuc, Bia
1663.581	1663	Hex5HexNAc4 + 1Na		0	1	Bia
1688.613	1688	Hex3dHex1HexNAc5 + 1Na		0	1	Fuc, Bis
1704.608	1704	Hex4HexNAc5 + 1Na		0	1	Bis
1743.581	1743	Hex8HexNAc2 + 1Na		0	1	Man
1751.597	1751	Hex5HexNAc3NeuAc1 + 1Na		0	1	Hyb, Sia
1757.585	1757	Hex4dHex1HexNAc3NeuAc1 + 2Na		0	2	Fuc, Sia

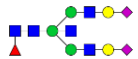
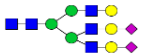
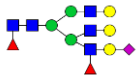
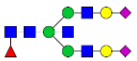
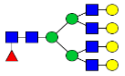
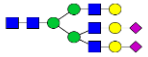
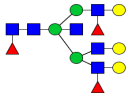
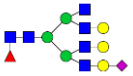
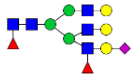
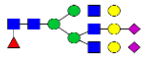
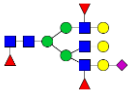
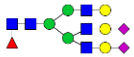
1773.579	1773	Hex5HexNAc3NeuAc1 + 2Na		0	2	Hyb, Sia
1809.639	1809	Hex5dHex1HexNAc4 + 1Na		0	1	Fuc, Bia
1814.606	1814	Hex4HexNAc4NeuAc1 + 2 Na		0	2	Bia, Sia
1815.559	1815	Hex4dHex1HexNAc3NeuAc1 + 1SO_4 + 1Na		1	1	Fuc, Sia, Sul
1825.634	1825 / 1826	Hex6HexNAc4 + 1Na		0	1	Bia, Alp
1831.554	1831	Hex5HexNAc3NeuAc1 + 1SO_4 + 1Na		1	1	Hyb, Sia, Sul
1850.666	1850	Hex4dHex1HexNAc5 + 1Na		0	1	Fuc, Bis
1853.536	1853	Hex5HexNAc3NeuAc1 + 1SO_4 + 2Na		1	2	Hyb, Sia, Sul
1866.661	1866	Hex5HexNAc5 + 1Na		0	1	Bis
1875.518	1875	Hex5HexNAc3NeuAc1 + 1SO_4 + 3Na		1	3	Hyb, Sia, Sul
1891.692	1891	Hex3dHex1HexNAc6 + 1Na		0	1	Fuc, Tet
1905.634	1905 / 1906	Hex9HexNAc2 + 1Na		0	1	Man
1911.578	1910 / 1911	Hex5dHex1HexNAc4 + 1SO4 + 2Na		1	2	Fuc, Bia, Sul

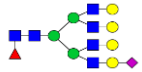
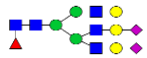
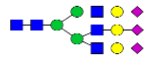
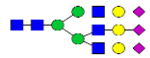
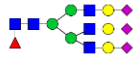
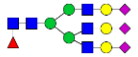
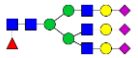
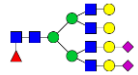
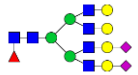
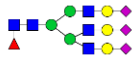
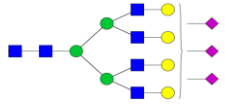
1935.632	1954 / 1955	Hex6HexNAc3NeuAc1 + 2Na		0	2	Hyb, Sia
1960.664	1960	Hex4dHex1HexNAc4NeuAc1 + 2Na		0	2	Fuc, Bia, Sia
1976.659	1976	Hex5HexNAc4NeuAc1 + 2Na		0	2	Bia, Sia
1996.724	1996	Hex4dHex2HexNAc5 + 1Na		0	1	Fuc, Bis
2012.719	2012	Hex5dHex1HexNAc5 + 1Na		0	1	Fuc, Bis
2018.639	2018	Hex4dHex1HexNAc4NeuAc1 + 1SO_4 + 1Na		1	1	Fuc, Bia, Sia, Sul
2028.714	2028	Hex6HexNAc5 + 1Na		0	1	Tri
2037.750	2037	Hex3dHex2HexNAc6 + 1Na		0	1	Fuc, Tet
2056.616	2056 / 2057	Hex5HexNAc4NeuAc1 + 1SO_4 + 2Na		1	2	Bia, Sia, Sul
2062.624	2062	Hex4dHex1HexNAc4NeuAc1 + 1SO_4 + 3Na		1	3	Fuc, Bia, Sia, Sul
2067.654	2067 / 2068	Hex10HexNAc2 + 1Na		0	1	Man
2100.735	2100	Hex5dHex1HexNAc4NeuAc1 + 1Na		0	1	Fuc, Bia, Sia
2122.717	2122	Hex5dHex1HexNAc4NeuAc1 + 2Na		0	2	Fuc, Bia, Sia

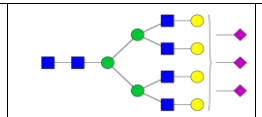
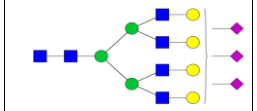
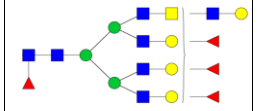
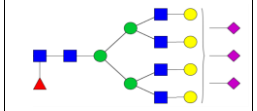
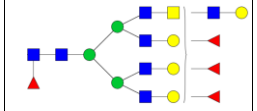
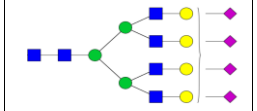
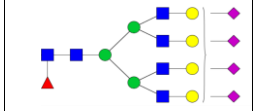
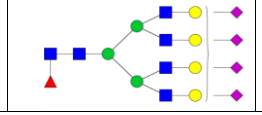
2157.756	2157	Hex5HexNAc5NeuAc1 + 1Na		0	1	Bis, Sia
2158.777	2158	Hex5dHex2HexNAc5 + 1Na		0	1	Fuc, Bis
2163.743	2163	Hex4dHex1HexNAc5NeuAc1 + 2Na		0	2	Fuc, Bis, Sia
2174.772	2174	Hex6dHex1HexNAc5 + 1Na		0	1	Fuc, Tri
2180.692	2180	Hex5dHex1HexNAc4NeuAc1 + 1Na		1	1	Fuc, Bia, Sia, Sul
2215.798	2215	Hex5dHex1HexNAc6 + 1Na		0	1	Fuc, Tet
2221.753	2221	Hex4dHex1HexNAc5NeuAc1 + $1SO_4$ + 1Na		1	1	Fuc, Bia, Sia, Sul
2231.793	2231	Hex6HexNAc6 + 1Na		0	1	Tet
2245.772	2245	Hex5HexNAc4NeuAc2 + 1Na		0	1	Bia, Sia
2259.738	2259	Hex8HexNAc3NeuAc1 + 2Na		0	2	Hyb, Sia
2267.754	2267	Hex5HexNAc4NeuAc2 + 2Na		0	2	Bia, Sia
2276.710	2275 / 2276	Hex6dHex1HexNAc5 + $1SO_4$ + 2Na		1	2	Fuc, Tri, Sul

2287.819	2287	Hex4dHex2HexNAc5NeuAc1 + 1Na		0	1	Fuc, Bis, Sia
2289.736	2289	Hex5HexNAc4NeuAc2 + 3Na		0	3	Bia, Sia
2303.814	2303	Hex5dHex1HexNAc5NeuAc1 + 1Na		0	1	Fuc, Bis, Sia
2304.835	2304	Hex5dHex3HexNAc5 + 1Na		0	1	Fuc, Bis
2319.809	2319	Hex6HexNAc5NeuAc1 + 1Na		0	1	Tri, Sia
2325.796	2325	Hex5dHex1HexNAc5NeuAc1 + 2Na		0	2	Fuc, Tri, Sia
2339.695	2339	Hex8HexNAc3NeuAc1 + 1SO_4 + 2Na		1	2	Hyb, Sia, Sul
2341.791	2341	Hex6HexNAc5NeuAc1 + 2Na		0	2	Tri, Sia
2361.677	2361 / 2362	Hex8HexNAc3NeuAc1 + 1SO_4 + 3Na		1	3	Hyb, Sia, Sul
2369.693	2369	Hex5HexNAc4NeuAc2 + 1SO_4 + 3Na		1	3	Bia, Sia, Sul
2377.851	2377	Hex6dHex1HexNAc6 + 1Na		0	1	Fuc, Tet
2391.830	2391	Hex5dHex1HexNAc4NeuAc2 + 1Na		0	1	Fuc, Bia, Sia
2393.846	2393	Hex7HexNAc6 + 1Na		0	1	Tet

2413.812	2413	Hex5dHex1HexNAc4NeuAc2 + 2Na		0	2	Fuc, Bia, Sia
2421.748	2422	Hex6HexNAc5NeuAc1 + 1SO_4 + 2Na		1	2	Tri, Sia, Sul
2435.794	2435	Hex5dHex1HexNAc4NeuAc2 + 3Na		0	3	Fuc, Bia, Sia
2465.867	2465	Hex6dHex1HexNAc5NeuAc1 + 1Na		0	1	Fuc, Tri, Sia
2466.887	2466	Hex6dHex3HexNAc5 + 1Na		0	1	Fuc, Tri
2487.849	2487 / 2488	Hex6dHex1HexNAc5NeuAc1 + 2Na		0	2	Fuc, Tri, Sia
2523.909	2523	Hex6dHex2HexNAc6 + 1Na		0	1	Fuc, Tet
2537.733	2537	Hex4dHex1HexNAc4NeuAc2 + 1SO_4 + 3Na		1	4	Fuc, Bia, Sia, Sul
2539.904	2539 / 2540	Hex7dHex1HexNAc6 + 1Na		0	1	Fuc, Tet
2544.870	2544	Hex6HexNAc6NeuAc1 + 2Na		0	2	Tet, Sia
2550.790	2550	Hex5HexNAc5NeuAc2 + 1SO_4 + 2Na		1	2	Bis, Sia, Sul
2594.894	2594	Hex5HexNAc5NeuAc2 + 1SO_4 + 4Na		1	4	Bis, Sia, Sul
2610.904	2610	Hex6HexNAc5NeuAc2 + 1Na		0	1	Tri, Sia

2616.892	2616	Hex5dHex1HexNAc5NeuAc2 + 2Na		0	2	Fuc, Bis, Sia
2632.886	2632	Hex6HexNAc5NeuAc2 + 2Na		0	2	Tri, Sia
2633.907	2633	Hex6dHex2HexNAc5NeuAc1 + 2Na		0	2	Fuc, Tri, Sia
2638.873	2638	Hex5dHex1HexNAc5NeuAc2 + 3Na		0	3	Fuc, Bis, Sia
2641.843	2642	Hex7dHex1HexNAc6 + 1SO_4 + 2Na		1	2	Fuc, Tet, Sul
2654.868	2654 / 2655	Hex6HexNAc5NeuAc2 + 3Na		0	3	Tri, Sia
2669.967	2669	Hex6dHex3HexNAc6 + 1Na		0	1	Fuc, Tet
2690.928	2691	Hex6dHex1HexNAc6NeuAc1 + 2Na		0	2	Fuc, Tet, Sia
2735.846	2735	Hex6dHex2HexNAc5NeuAc1 + 1SO_4 + 3Na		1	3	Fuc, Tri, Sia, Sul
2756.962	2756	Hex6dHex1HexNAc5NeuAc2 + 1Na		0	1	Fuc, Tri, Sia
2757.983	2757	Hex6dHex3HexNAc5NeuAc1 + 1Na		0	1	Fuc, Tri, Sia
2778.934	2778	Hex6dHex1HexNAc5NeuAc2 + 1Na		0	2	Fuc, Tri, Sia
2800.926	2800 / 2801	Hex6dHex1HexNAc5NeuAc2 + 3Na		0	3	Fuc, Tri, Sia

2852.981	2852 / 2853	Hex7dHex1HexNAc6NeuAc1 + 2Na		0	2	Fuc, Tet, Sia
2923.979	2923	Hex6dHex1HexNAc5NeuAc2 + 1SO4 + 4Na		1	4	Fuc, Tri, Sia, Sul
2945.964	2945	Hex6HexNAc5NeuAc3 + 3Na		0	3	Tri, Sia
2967.946	2967 / 2968	Hex6HexNAc5NeuAc3 + 4Na		0	4	Tri, Sia
3004.005	3004	Hex6dHex1HexNAc6NeuAc2 + 3Na		0	3	Bis, Fuc, Tri, Sia
3048.058	3048	Hex6dHex1HexNAc5NeuAc3 + 1Na		0	1	Fuc, Tri, Sia
3070.040	3070	Hex6dHex1HexNAc5NeuAc3 + 2Na		0	2	Fuc, Tri, Sia
3092.022	3092 / 3093	Hex6dHex1HexNAc5NeuAc3 + 3Na		0	3	Fuc, Tri, Sia
3114.004	3113 / 3114	Hex6dHex1HexNAc5NeuAc3 + 4Na		0	4	Fuc, Tri, Sia
3144.077	3144	Hex7dHex1HexNAc6NeuAc2 + 2Na		0	2	Fuc, Tet, Sia
3166.059	3166	Hex7dHex1HexNAc6NeuAc2 + 3Na		0	3	Fuc, Tet, Sia
3193.086	3193	Hex6dHex1HexNAc5NeuAc3 + 1SO4 + 3Na		1	2	Fuc, Tri, Sia, Sul
3267.137	3267	Hex7HexNAc6NeuAc3 + 2Na		0	2	Tri, Sia

3311.106	3311	Hex7HexNAc6NeuAc3 + 3Na		0	3	Tri, Sia
3333.074	3333	Hex7HexNAc6NeuAc3 + 4Na		0	4	Tri, Sia
3384.116	3384	Hex8HexNAc8Fuc4 + 1Na		0	1	Fuc,Tet
3479.136	3479	Hex7dHex1HexNAc6NeuAc3 + 4Na		0	4	Fuc, Tet, Sia
3486.141	3486	Hex8HexNAc8Fuc4 + 1SO4 + 2Na		1	2	Fuc,Tet, Sul
3646.247	3646	Hex7HexNAc6NeuAc4 + 5Na		0	5	Tet, Sia
3770.314	3770	Hex7dHex1HexNAc6NeuAc4 + 4Na		0	4	Fuc, Tet, Sia
3792.342	3792	Hex7dHex1HexNAc6NeuAc4 + 5Na		0	5	Fuc, Tet, Sia

Fuc = fucosylation, Sia = sialylation, Man = high-mannose, Hyb = hybrid, Bia = biantennary, Bis = bisecting, Tri = triantennary, Tet = tetraantennary, Sul = sulfation, Alp = alpha-galactose. The number of sulfates present is indicated in a separate column (+SO4). Glycosylation Class = class(es) each glycan was included for Fig. 2, Fig. 4, and Tables 2, 3, S2, S3.

Table S2. Mean difference and 95% confidence interval in the relative frequencies of the urine glycans.

Peak	LN vs. Healthy (95% CI)	Q-value	Male vs. Female (95% CI)	Q-value	Peak	LN vs. Healthy (95% CI)	Q-value	Male vs. Female (95% CI)	Q-value
1419	-1.054 (-1.331 ,-0.777)	3.88E-07	-0.321 (-0.598 ,-0.044)	0.2004	**3646**	0.065 (0.023 ,0.107)	0.0093	-0.024 (-0.066 ,0.018)	0.5290
1853	-0.441 (-0.554 ,-0.328)	3.88E-07	0.003 (-0.11 ,0.116)	0.9770	**3770**	-0.031 (-0.05 ,-0.011)	0.0094	-0.008 (-0.028 ,0.011)	0.6218
2122	-2.637 (-3.323 ,-1.95)	3.88E-07	-0.714 (-1.4 ,-0.027)	0.2236	**sulfated**	-0.74 (-1.229 ,-0.251)	0.0105	0.51 (0.021 ,0.999)	0.2236
2361*	0.345 (0.258 ,0.432)	3.88E-07	0.28 (0.193 ,0.367)	4.00E-05	**2413**	-0.281 (-0.469 ,-0.092)	0.0118	-0.055 (-0.243 ,0.134)	0.7160
1831	-0.416 (-0.534 ,-0.298)	1.41E-06	-0.007 (-0.125 ,0.111)	0.9381	**1444**	-0.065 (-0.11 ,-0.021)	0.0125	-0.044 (-0.089 ,0)	0.2509
mannose	-2.796 (-3.609 ,-1.984)	1.93E-06	-0.977 (-1.79 ,-0.165)	0.2004	**1850**	-0.537 (-0.911 ,-0.164)	0.0144	-0.398 (-0.772 ,-0.025)	0.2236
1581	-0.734 (-0.957 ,-0.51)	3.55E-06	-0.316 (-0.539 ,-0.092)	0.1395	**2391**	-0.089 (-0.152 ,-0.027)	0.0151	-0.018 (-0.081 ,0.044)	0.7160
2967	1.445 (1.006 ,1.884)	3.55E-06	0.289 (-0.15 ,0.728)	0.4887	**3093**	0.096 (0.028 ,0.164)	0.0156	0.008 (-0.06 ,0.076)	0.8852
2174	-0.452 (-0.596 ,-0.308)	7.57E-06	-0.166 (-0.31 ,-0.022)	0.2004	**3113**	0.264 (0.077 ,0.45)	0.0156	0.156 (-0.031 ,0.342)	0.3481
1257	-0.42 (-0.555 ,-0.285)	7.92E-06	-0.164 (-0.299 ,-0.029)	0.2004	**1647**	1.491 (0.424 ,2.557)	0.0165	-0.101 (-1.168 ,0.965)	0.9129
2289*	5.116 (3.351 ,6.881)	2.21E-05	3.184 (1.419 ,4.949)	0.0439	**1954**	0.568 (0.152 ,0.984)	0.0191	0.292 (-0.124 ,0.708)	0.4660
2377	-0.606 (-0.814 ,-0.397)	2.21E-05	-0.236 (-0.444 ,-0.027)	0.2004	**bisecting**	-2.236 (-3.916 ,-0.557)	0.0216	-1.296 (-2.976 ,0.383)	0.3938
2012	-1.008 (-1.373 ,-0.644)	4.28E-05	-0.463 (-0.827 ,-0.098)	0.2004	**3092**	0.064 (0.016 ,0.111)	0.0216	0.01 (-0.037 ,0.058)	0.7953
2056	-0.235 (-0.321 ,-0.149)	4.69E-05	-0.044 (-0.13 ,0.042)	0.5766	**1079**	-0.209 (-0.37 ,-0.048)	0.0252	-0.188 (-0.349 ,-0.027)	0.2004
2221	0.144 (0.091 ,0.198)	5.72E-05	0.07 (0.017 ,0.124)	0.1955	**2638**	0.347 (0.078 ,0.616)	0.0255	-0.094 (-0.363 ,0.175)	0.6833
1866	-0.262 (-0.367 ,-0.158)	0.0001	-0.117 (-0.221 ,-0.012)	0.2033	**1611**	0.063 (0.014 ,0.112)	0.0264	0.031 (-0.018 ,0.08)	0.5037
tetraantennary	-1.494 (-2.074 ,-0.914)	0.0001	-0.249 (-0.829 ,0.331)	0.6218	**3004**	-0.062 (-0.111 ,-0.013)	0.0275	-0.02 (-0.069 ,0.029)	0.6461
1996	-0.109 (-0.152 ,-0.067)	0.0001	-0.009 (-0.052 ,0.033)	0.7953	**2028**	-0.072 (-0.128 ,-0.015)	0.0287	-0.09 (-0.146 ,-0.033)	0.1114
2669	-0.367 (-0.509 ,-0.225)	0.0001	0.002 (-0.14 ,0.144)	0.9781	**1773**	0.516 (0.094 ,0.937)	0.0323	-0.156 (-0.577 ,0.266)	0.6787
1891	-0.31 (-0.439 ,-0.181)	0.0002	-0.038 (-0.167 ,0.091)	0.7160	**3384**	-0.034 (-0.063 ,-0.006)	0.0323	-0.014 (-0.043 ,0.014)	0.5766
2158	-0.564 (-0.797 ,-0.331)	0.0002	-0.059 (-0.292 ,0.174)	0.7656	**2594**	0.024 (0.004 ,0.044)	0.0346	0.012 (-0.008 ,0.032)	0.5239
2852	-0.155 (-0.22 ,-0.089)	0.0002	-0.062 (-0.127 ,0.004)	0.2686	**3193**	-0.032 (-0.06 ,-0.005)	0.0346	-0.013 (-0.041 ,0.014)	0.5863
3333	0.175 (0.104 ,0.246)	0.0002	-0.026 (-0.097 ,0.045)	0.6787	**1875**	-0.029 (-0.054 ,-0.005)	0.0353	-0.003 (-0.028 ,0.021)	0.8724
1809	-1.532 (-2.191 ,-0.873)	0.0003	-0.684 (-1.343 ,-0.025)	0.2236	**2341**	0.214 (0.03 ,0.398)	0.0401	-0.061 (-0.245 ,0.123)	0.6992
2267	1.34 (0.762 ,1.919)	0.0003	0.603 (0.025 ,1.182)	0.2236	**1751**	0.132 (0.012 ,0.252)	0.0526	-0.094 (-0.214 ,0.026)	0.3903
2632	0.41 (0.233 ,0.588)	0.0003	-0.062 (-0.24 ,0.115)	0.6833	**1837**	-0.091 (-0.176 ,-0.005)	0.0625	0.062 (-0.023 ,0.148)	0.4360
1743	-0.277 (-0.402 ,-0.153)	0.0005	-0.073 (-0.198 ,0.051)	0.5239	**1298**	-0.026 (-0.052 ,-0.001)	0.0650	-0.004 (-0.03 ,0.021)	0.8502
2100	-0.674 (-0.983 ,-0.365)	0.0005	-0.158 (-0.467 ,0.151)	0.5766	**1501**	0.065 (0.001 ,0.129)	0.0736	0.016 (-0.048 ,0.08)	0.7656
2245	0.795 (0.432 ,1.158)	0.0005	0.298 (-0.065 ,0.661)	0.3481	**2757**	-0.041 (-0.087 ,0.004)	0.1056	-0.053 (-0.099 ,-0.008)	0.2004
2487	-0.567 (-0.826 ,-0.309)	0.0005	-0.243 (-0.502 ,0.015)	0.2686	**1460**	0.142 (-0.015 ,0.299)	0.1096	-0.098 (-0.255 ,0.059)	0.5037
2945	0.3 (0.16 ,0.44)	0.0007	-0.008 (-0.148 ,0.132)	0.9381	**2026**	-0.037 (-0.078 ,0.005)	0.1182	0.02 (-0.021 ,0.062)	0.5863
sialylated	6.129 (3.082 ,9.176)	0.0011	3.448 (0.401 ,6.495)	0.2004	**3166**	-0.074 (-0.161 ,0.012)	0.1271	-0.026 (-0.113 ,0.06)	0.7160
1485	2.221 (1.117 ,3.325)	0.0011	0.165 (-0.939 ,1.269)	0.8689	**1757**	-0.03 (-0.065 ,0.006)	0.1331	-0.021 (-0.056 ,0.015)	0.5239
1704	-0.105 (-0.157 ,-0.054)	0.0011	-0.026 (-0.078 ,0.025)	0.5766	**2616**	0.035 (-0.011 ,0.081)	0.1765	-0.035 (-0.081 ,0.011)	0.3981
2465	-0.128 (-0.19 ,-0.066)	0.0011	-0.058 (-0.12 ,0.004)	0.2686	**1910**	-0.092 (-0.224 ,0.04)	0.2190	0.048 (-0.084 ,0.18)	0.6787
2523	-0.18 (-0.269 ,-0.091)	0.0011	-0.041 (-0.13 ,0.048)	0.6104	**2433**	-0.024 (-0.066 ,0.017)	0.2992	-0.019 (-0.061 ,0.022)	0.6063
2654	0.822 (0.414 ,1.23)	0.0011	0.006 (-0.402 ,0.414)	0.9781	**3792**	-0.054 (-0.145 ,0.037)	0.2992	-0.015 (-0.106 ,0.076)	0.8616
2287	0.378 (0.186 ,0.57)	0.0014	0.276 (0.084 ,0.468)	0.1395	**1688**	0.137 (-0.098 ,0.373)	0.3028	-0.088 (-0.323 ,0.148)	0.6787
2435	-1.108 (-1.668 ,-0.547)	0.0014	-0.076 (-0.637 ,0.484)	0.8724	**2800**	-0.181 (-0.513 ,0.151)	0.3371	-0.097 (-0.429 ,0.235)	0.7160
1663	-0.799 (-1.214 ,-0.384)	0.0017	0.031 (-0.384 ,0.446)	0.9370	**biantennary**	1.154 (-1.402 ,3.71)	0.4321	3.045 (0.489 ,5.601)	0.2004
2383	0.035 (0.017 ,0.053)	0.0019	0.027 (0.009 ,0.045)	0.1395	**3479**	-0.025 (-0.08 ,0.03)	0.4321	-0.018 (-0.073 ,0.037)	0.6992
1905	-0.31 (-0.482 ,-0.139)	0.0030	-0.1 (-0.272 ,0.071)	0.5239	**1815**	0.016 (-0.02 ,0.052)	0.4401	0.032 (-0.004 ,0.068)	0.3143
3144	-0.045 (-0.07 ,-0.02)	0.0035	-0.021 (-0.046 ,0.004)	0.3481	**2544**	-0.025 (-0.087 ,0.037)	0.4758	0.06 (-0.002 ,0.122)	0.2658
2339*	0.264 (0.114 ,0.415)	0.0037	0.343 (0.193 ,0.494)	0.0046	**2303**	-0.034 (-0.123 ,0.054)	0.4893	-0.058 (-0.146 ,0.031)	0.4887
2393	-0.135 (-0.211 ,-0.059)	0.0037	-0.053 (-0.129 ,0.023)	0.4677	**3048**	0.014 (-0.024 ,0.053)	0.5003	0.017 (-0.021 ,0.056)	0.6104
2923	0.198 (0.085 ,0.311)	0.0038	-0.018 (-0.131 ,0.095)	0.8616	**2325**	-0.09 (-0.421 ,0.24)	0.6366	-0.128 (-0.459 ,0.202)	0.6707
2610	0.223 (0.092 ,0.355)	0.0050	-0.026 (-0.157 ,0.106)	0.8317	**3070**	-0.005 (-0.026 ,0.015)	0.6420	-0.013 (-0.034 ,0.007)	0.4887
triantennary	2.608 (1.056 ,4.16)	0.0053	-0.509 (-2.061 ,1.043)	0.6992	**2778**	-0.025 (-0.126 ,0.076)	0.6612	-0.044 (-0.145 ,0.057)	0.6218
1960	0.082 (0.033 ,0.131)	0.0054	-0.006 (-0.055 ,0.043)	0.8852	**1976**	-0.147 (-0.977 ,0.683)	0.7589	0.46 (-0.37 ,1.29)	0.5445
fucosylated	-7.175 (-11.564 ,-2.786)	0.0062	-3.621 (-8.01 ,0.768)	0.3481	**2537**	-0.006 (-0.042 ,0.03)	0.7693	-0.034 (-0.07 ,0.002)	0.2686
2304	-0.264 (-0.425 ,-0.103)	0.0062	-0.069 (-0.23 ,0.092)	0.6218	**1825**	0.008 (-0.057 ,0.073)	0.8251	-0.06 (-0.125 ,0.005)	0.2686
2319	0.099 (0.038 ,0.16)	0.0062	-0.039 (-0.1 ,0.022)	0.4924	**2756**	0.001 (-0.026 ,0.027)	0.9799	0.018 (-0.009 ,0.044)	0.4887
1814	0.048 (0.018 ,0.079)	0.0080	0.014 (-0.016 ,0.045)	0.6063	**hybrid**	0.009 (-0.854 ,0.871)	0.9847	0.051 (-0.812 ,0.913)	0.9381
2163	-0.334 (-0.546 ,-0.121)	0.0082	-0.122 (-0.334 ,0.091)	0.5290					

Mean difference and 95% confidence interval in the relative frequencies of the urine glycans or glycan groups between LN and HC and between females and males were calculated. Q-values reported are the Benjamini-Hochberg FDR corrected values for (1) the difference between HC and LN and (2) between males and females. Values in grey are for those peaks that were not significantly different between HC and LN after FDR correction. *N-glycans significantly different between females and males.

Table S3. Mean difference and 95% confidence interval in the relative frequencies of the serum glycans.

Peak	LN vs. Healthy (95% CI)	Q-value	Male vs. Female (95% CI)	Q-value	Peak	LN vs. Healthy (95% CI)	Q-value	Male vs. Female (95% CI)	Q-value
high mannose	-1.147 (-1.546 ,-0.749)	0.0002	-0.011 (-0.41 ,0.387)	0.9693	2801	0.07 (-0.016 ,0.157)	0.2310	0.033 (-0.053 ,0.12)	0.6634
sulfated	1.63 (1.052 ,2.207)	0.0002	0.298 (-0.28 ,0.875)	0.5584	1688	0.146 (-0.036 ,0.327)	0.2351	-0.093 (-0.275 ,0.088)	0.5584
triantennary	3.313 (2.028 ,4.598)	0.0004	-0.608 (-1.893 ,0.677)	0.5584	1079	-0.012 (-0.027 ,0.004)	0.2652	-0.009 (-0.024 ,0.007)	0.5584
2275	0.733 (0.429 ,1.036)	0.0007	-0.232 (-0.536 ,0.071)	0.4529	1298	0.143 (-0.052 ,0.338)	0.2851	0.26 (0.065 ,0.455)	0.4132
1419	-0.305 (-0.433 ,-0.177)	0.0007	-0.008 (-0.136 ,0.12)	0.9693	2174	0.254 (-0.094 ,0.602)	0.2851	0.225 (-0.123 ,0.573)	0.5243
1743	-0.224 (-0.319 ,-0.13)	0.0007	-0.04 (-0.135 ,0.054)	0.6066	2466	0.028 (-0.011 ,0.066)	0.2936	-0.013 (-0.052 ,0.025)	0.6886
1905	-0.318 (-0.454 ,-0.183)	0.0007	0.028 (-0.108 ,0.163)	0.8697	hybrid	-0.476 (-1.182 ,0.23)	0.3284	-0.016 (-0.722 ,0.69)	0.9693
1809	-2.168 (-3.099 ,-1.237)	0.0007	-0.586 (-1.517 ,0.345)	0.5252	2422	0.186 (-0.105 ,0.478)	0.3600	0.15 (-0.141 ,0.442)	0.5584
1581	-0.115 (-0.167 ,-0.064)	0.0011	-0.005 (-0.057 ,0.046)	0.9582	2259	0.133 (-0.081 ,0.346)	0.3745	-0.142 (-0.356 ,0.071)	0.5243
2028	0.858 (0.439 ,1.278)	0.0025	-0.318 (-0.738 ,0.101)	0.4529	3114	0.021 (-0.016 ,0.058)	0.4285	0.018 (-0.019 ,0.055)	0.5584
2523	0.218 (0.108 ,0.329)	0.0035	-0.1 (-0.211 ,0.01)	0.4473	1501	0.066 (-0.054 ,0.186)	0.4481	0.162 (0.042 ,0.282)	0.4132
tetraantennary	0.493 (0.235 ,0.751)	0.0045	-0.243 (-0.501 ,0.015)	0.4473	1460	-0.253 (-0.718 ,0.213)	0.4481	-0.012 (-0.477 ,0.454)	0.9693
2540	0.113 (0.051 ,0.176)	0.0073	-0.008 (-0.07 ,0.055)	0.9482	2012	-0.395 (-1.143 ,0.353)	0.4591	-0.264 (-1.012 ,0.484)	0.6886
2968	0.094 (0.041 ,0.146)	0.0076	-0.039 (-0.092 ,0.013)	0.4529	2180	0.016 (-0.015 ,0.046)	0.4762	-0.014 (-0.045 ,0.016)	0.5584
1136	0.111 (0.048 ,0.173)	0.0076	0.059 (-0.004 ,0.121)	0.4473	2267	0.056 (-0.055 ,0.166)	0.4762	0.098 (-0.013 ,0.208)	0.4473
2633	0.095 (0.041 ,0.15)	0.0076	-0.045 (-0.099 ,0.01)	0.4529	2100	-0.043 (-0.131 ,0.045)	0.4841	-0.007 (-0.095 ,0.081)	0.9693
2319	0.125 (0.054 ,0.197)	0.0079	-0.073 (-0.145 ,-0.002)	0.4473	1825	-0.072 (-0.23 ,0.085)	0.5191	-0.166 (-0.324 ,-0.009)	0.4473
1444	-0.211 (-0.334 ,-0.088)	0.0081	-0.055 (-0.178 ,0.068)	0.5854	1850	-0.111 (-0.358 ,0.137)	0.5290	-0.131 (-0.378 ,0.117)	0.5584
2655	0.234 (0.098 ,0.371)	0.0081	-0.093 (-0.229 ,0.044)	0.5243	1606	-0.023 (-0.074 ,0.029)	0.5315	-0.01 (-0.061 ,0.042)	0.8743
2231	-0.059 (-0.095 ,-0.023)	0.0102	-0.026 (-0.062 ,0.01)	0.4731	2369	0.014 (-0.019 ,0.047)	0.5355	-0.008 (-0.041 ,0.025)	0.8161
2341	0.426 (0.168 ,0.684)	0.0102	-0.225 (-0.483 ,0.033)	0.4473	2037	-0.007 (-0.026 ,0.011)	0.5579	-0.009 (-0.028 ,0.009)	0.5584
1647	-1.716 (-2.799 ,-0.632)	0.0141	-0.604 (-1.688 ,0.479)	0.5584	933	-0.015 (-0.051 ,0.022)	0.5670	-0.006 (-0.043 ,0.03)	0.8756
2393	0.208 (0.067 ,0.35)	0.0234	-0.118 (-0.26 ,0.023)	0.4529	2550	-0.011 (-0.039 ,0.017)	0.5670	0.015 (-0.013 ,0.043)	0.5584
1257	-0.101 (-0.173 ,-0.028)	0.0351	0.017 (-0.055 ,0.09)	0.8161	1976	0.247 (-0.426 ,0.92)	0.5865	0.731 (0.058 ,1.404)	0.4473
2122	-0.498 (-0.875 ,-0.121)	0.0449	0.053 (-0.324 ,0.43)	0.9252	2413	0.008 (-0.015 ,0.032)	0.5865	-0.027 (-0.051 ,-0.004)	0.4473
biantennary	-2.061 (-3.635 ,-0.486)	0.0458	1.46 (-0.114 ,3.035)	0.4473	1704	0.011 (-0.021 ,0.044)	0.5885	-0.016 (-0.049 ,0.016)	0.5584
1095	-0.083 (-0.152 ,-0.015)	0.0644	-0.001 (-0.07 ,0.067)	0.9693	2435	-0.028 (-0.117 ,0.06)	0.6283	-0.016 (-0.105 ,0.072)	0.8743
2157	0.12 (0.022 ,0.219)	0.0644	0.088 (-0.011 ,0.186)	0.4473	2018	-0.008 (-0.036 ,0.019)	0.6293	-0.02 (-0.048 ,0.007)	0.4529
2303	0.055 (0.009 ,0.102)	0.0721	-0.036 (-0.082 ,0.011)	0.4529	2325	-0.098 (-0.418 ,0.221)	0.6293	-0.161 (-0.481 ,0.158)	0.5584
1910	0.555 (0.084 ,1.026)	0.0721	0.544 (0.073 ,1.015)	0.4473	1663	0.386 (-0.908 ,1.681)	0.6346	1.135 (-0.16 ,2.429)	0.4473
1954	0.152 (0.003 ,0.302)	0.1339	0.124 (-0.025 ,0.274)	0.4529	2289	0.105 (-0.254 ,0.464)	0.6350	0.467 (0.108 ,0.826)	0.4132
1339	0.087 (0.001 ,0.173)	0.1339	0.026 (-0.06 ,0.112)	0.7405	1282	-0.027 (-0.124 ,0.07)	0.6479	-0.065 (-0.162 ,0.032)	0.5243
sialylated	1.661 (0.018 ,3.304)	0.1339	0.825 (-0.818 ,2.468)	0.5584	2062	0.005 (-0.016 ,0.026)	0.6979	-0.008 (-0.029 ,0.013)	0.6636
fucosylated	-2.425 (-4.909 ,0.058)	0.1495	-1.939 (-4.423 ,0.544)	0.4529	1622	-0.002 (-0.016 ,0.011)	0.7630	-0.006 (-0.02 ,0.007)	0.5584
1485	0.829 (-0.041 ,1.7)	0.1594	-0.459 (-1.33 ,0.411)	0.5584	2245	0.013 (-0.061 ,0.087)	0.7630	0.022 (-0.052 ,0.096)	0.7405
1866	0.067 (-0.006 ,0.141)	0.1745	-0.024 (-0.098 ,0.049)	0.7029	2056	-0.037 (-0.252 ,0.177)	0.7630	0.015 (-0.2 ,0.229)	0.9693
2735	0.046 (-0.005 ,0.097)	0.1745	0.001 (-0.05 ,0.052)	0.9693	1611	-0.003 (-0.024 ,0.018)	0.8019	0.021 (0 ,0.042)	0.4473
1773	-0.197 (-0.415 ,0.02)	0.1745	0.013 (-0.205 ,0.23)	0.9693	2537	-0.003 (-0.033 ,0.027)	0.8552	-0.019 (-0.049 ,0.011)	0.5243
2488	0.144 (-0.022 ,0.309)	0.1984	0.004 (-0.161 ,0.17)	0.9693	bisecting	-0.084 (-1.556 ,1.388)	0.9116	-0.767 (-2.239 ,0.705)	0.5584
1960	-0.023 (-0.052 ,0.005)	0.2300	0.019 (-0.01 ,0.047)	0.5243					

Mean difference and 95% confidence interval in the relative frequencies of the serum glycans or glycan groups between LN and HC and between females and males were calculated. Q-values reported are the Benjamini-Hochberg FDR corrected values for (1) the difference between HC and LN and (2) between males and females. Values in grey are for those peaks that were not significantly different between HC and LN after FDR correction. None of the glycans were significantly different between females and males.

ACR Abstract; 2023

Association of Biologic Sex, Glycosphingolipids, and the N-glycome in Lupus Nephritis and Renal Mesangial Cell Function

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Background: SLE primarily afflicts women and many SLE patients develop nephritis, a serious complication of lupus. Identification of biomarkers and the pathogenic mechanisms underlying LN is crucial to better understand sex bias and disease progression. Therefore, we interrogated glycosphingolipid (GSL) metabolism and the N-glycome with respect to disease and biologic sex in LN patient samples. We also evaluated response of human primary renal mesangial cells (hRMCs) with respect to disease and biologic sex.

Methods: Urine and serum were collected from 20 healthy control (HC) subjects and 20 LN patients who met the ACR criteria for active disease. Ten males and 10 females were included in each group. N-linked glycans attached to proteins (N-glycans) were measured by matrix-assisted laser desorption/ionization quadrupole time of flight (MALDI-QTOF) and the GSL lactosylceramide (LacCer) was quantified by Supercritical fluid chromatography-tandem mass spectrometry (SFC-MS/MS). Responses of male- and female-derived hRMCs to treatment with sera from each LN or HC subject were assessed by measuring intracellular calcium (Ca^{2+}) using fluor8 indicator, cytokine secretion by ELISA, GSLs levels by SFC-MS/MS, and N-glycans by MALDI-QTOF. Associations between N-glycans with LN status and biologic sex were evaluated using linear mixed models. P-values were adjusted using False Discover Rate with <0.05 considered meaningful.

Results: All major LacCer species and total LacCers were significantly elevated and 72 urine N-glycans were significantly altered in LN patients compared to HCs. In particular, there was a significant increase in the N-glycans associated with pro-inflammatory IgGs and a decrease in N-glycans associated with anti-inflammatory IgGs in the urine of LN patients. The increase in urine LacCers (LN vs HC) was 2-3 fold higher in males, and three urine N-glycans differed significantly between the sexes. Three individual N-glycans provided perfect separation of LN and HC (AUC of 1.0) when added to a model that included only total LacCers and biologic sex. In the serum, 2 of the major LacCer species and 21 N-glycans were significantly altered in LN compared to HC. *In vitro*, no differences were observed in the responses of hRMCs to female- vs male-derived sera. However, the female-derived hRMCs exhibited significantly higher Ca^{2+} flux and cytokine secretion compared to male-derived hRMCs in response to LN sera. The female-derived hRMCs expressed higher levels of GSLs than the male-derived hRMCs.

Conclusions: Urine LacCers levels and changes in the N-glycome may serve as robust biomarkers of LN and likely reflect renal disease activity. The larger increase in LacCers in LN males compared to LN females may underscore worse renal disease in males once tolerance is broken. The significantly higher levels of LacCers observed in the female-derived hRMCs may partly explain the heightened pathologic response. Thus, elevated GSL metabolism in females may poise renal cells to be more sensitive to or respond more robustly to inflammatory stimuli.

Keywords:
glycosylation, N-glycan, glycosphingolipid, lactosylceramide, hexosylceramide, lupus nephritis, mesangial cell, sex bias, biomarker

Identifying urine N-glycans as potential biomarkers of Lupus Nephritis

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Lupus Nephritis (LN) is a type of autoimmune kidney disease in the context of Systemic Lupus Erythematosus (SLE), impacting roughly 50% of SLE patients. SLE is a multi-system autoimmune disease predominantly affecting women of childbearing age. Diagnosis relies on clinical observations supported by lab studies and regular screenings such as urinalyses and glomerular filtration rate estimation, which are integral components of ongoing SLE and LN patient management.

Given the complexity of LN and renal biopsy being the gold standard for diagnostics and prognostication, a less invasive urine-based diagnostic approach holds immense promise. The current study sought to address this by comparing N-glycans derived from glycoproteins in urine samples from individuals with LN, healthy controls (HC), and those with SLE without LN.

The underlying hypothesis was that distinct N-glycan profiles associated with LN can be detected in urine, thus providing potential diagnostic biomarkers. Here, we analyzed 120 de-identified urine samples from the MUSC Biorepository: 40 LN, 40 HC and 40 SLE samples. Utilizing established urine processing methods, urine was concentrated and spotted in duplicate onto amine reactive slides for PNGase digestion to release N-glycans. Detection was performed by MALDI-QTOF mass spectroscopy and data analyzed using SCILS software.

Our findings indicate that N-glycan profiles demonstrated the sensitivity required to differentiate LN from HC and SLE samples using one-way ANOVA analysis. A total of 87 unique N-glycans were identified, and of those, 30 were statistically significant using a p-value of 0.00005. In LN urine samples, sialylated and fucosylated triantennary N-glycans were particularly elevated. Furthermore, N-glycans associated with immunoglobulin G (IgG) displayed significant elevation in LN samples when compared to both HC and SLE samples.

This study underscores the importance of urine glycomic profiles as a promising avenue for refining diagnostic procedures and uncovering novel biomarkers specific to diseases like Lupus Nephritis.